THE IMMUNE RESPONSE TO MURINE RETROVIRAL INFECTION

by
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STATEMENT

The experiments described in this thesis represent my own work except for the following. Samples were processed for histology and stained by Sheila Cook. Sabin Gruninger and Geoff Osbourne configured and operated the FACS.

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When I first submitted this thesis I wrote this -

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ABSTRACT

The growth of Rauscher murine leukaemia virus (R-MuLV) in mice is regulated by a number of autosomal genes. These genes influence virus replication or control the antiviral immune response generated toward R-MuLV. The experiments described in this thesis were designed to examine the role of the immune response in resistance or susceptibility to R-MuLV infection.

In agreement with previous reports, BALB/c mice were found to be highly susceptible to R-MuLV infection. High levels of infectious virus and virus antigen were present in plasma and spleen of infected mice for at least 3 weeks post infection. In contrast, C57BL/6 mice controlled R-MuLV with little viral antigen or infectious virus present in plasma or spleen by 3 weeks post infection.

The ability of spleen T and B cells from R-MuLV infected mice to proliferate following polyclonal stimulation *in vitro* was markedly impaired following R-MuLV infection. This effect was more pronounced in BALB/c mice compared with C57BL/6 mice. In contrast, R-MuLV infection had little effect upon lymph node T cell proliferation. Investigation of the mechanism(s) which lead to immunosuppression suggested that suppressed lymphocyte proliferation may not be due to decreased proportion of T or B cells in the spleen of infected mice. The proliferation of spleen cells from uninfected BALB/c or C57BL/6 mice was suppressed by co-culture with splenocytes from infected mice. This observation suggested that a suppressive factor was produced by spleen cells from R-MuLV infected mice. Further studies showed that immunosuppressive molecules nitric oxide or prostaglandins were not involved in impaired lymphocyte proliferation.

The cytokines present during the generation of an immune response are integral to determining the character of the response. As certain immune responses correlate with susceptibility or resistance to infectious agents cytokine expression during R-MuLV infection of BALB/c and C57BL/6 mice was examined. Analysis of cytokine mRNA in the spleen and production of cytokines by spleen and lymph node cells following

restimulation *in vitro* with anti-CD3 antibodies showed that both BALB/c and C57BL/6 mice produced predominantly type 1 cytokines following R-MuLV infection. This suggested that inappropriate cytokine production was unlikely to be responsible for susceptibility to R-MuLV exhibited by BALB/c mice. However, spleen cells from C57BL/6 mice produced higher levels of IFN-γ compared with cells from BALB/c mice which raised the possibility that insufficient IFN-γ production may lead to an ineffective immune response in BALB/c mice. The role of cytokines in resistance or susceptibility to R-MuLV was examined by treating infected mice with cytokine neutralising antibodies. Treatment of BALB/c or C57BL/6 mice with antibodies that neutralised IL-2, IL-4, IL-6, IL-10, IFN-γ or TNF-α during R-MuLV infection suggested that individually these factors (including IFN-γ) were not essential for resistance or susceptibility to R-MuLV. Mice lacking functional IFN-γ receptors appeared to control R-MuLV infection while wild type mice did not, which suggests that IFN-γ may be involved in susceptibility to R-MuLV infection in 129/Sv mice. Analysis of the role of T cell subpopulation in disease associated with R-MuLV infection suggested that in both BALB/c and C57BL/6 mice $CD4^+$ T cells may be required for full development of spleomegaly induced by R-MuLV.

Since the role of NO in immunity to retroviral infection is largely unknown, the involvement of NO in resistance or susceptibility to R-MuLV was examined. Similar levels of mRNA encoding the NO producing enzyme inducible NO synthase in spleen cells of both BALB/c and C57BL/6 mice following R-MuLV infection. The magnitude and time of induction were largely equivalent. Spleen cells from infected mice were also found to produce NO following *in vitro* restimulation with LPS or anti-CD3 antibodies. Inhibition of NO production, however failed to affect virus growth *in vivo*. Taken together, these observations suggest that NO does not play a major role in the pathogenesis of R-MuLV in susceptible or resistant mice.

ABBREVIATIONS

APC	Antigen presenting cell
BSA	Bovine serum albumin
CMI	Cell mediated immunity
Con A	Concanavalin A
CPM	Counts per minute
CTL	Cytotoxic T lymphocyte
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DTH	Delayed type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
eco	Ecotropic
F-MuLV	Friend murine leukaemia virus
g	Relative centrifugal force
GM-CSF	Granulocyte-macrophage colony stimulating factor
h	Hours
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
HPRT	Hypoxyl ribonucleotidyl reductase
iNOS	Inducible nitric oxide synthase
i.p.	Intraperitoneal
i.v.	Intravenous
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
LT	Lymphotoxin
M	Molar
mAb	Monoclonal antibody
mRNA	Message ribonucleic acid
MCF	Mink cell focus forming
MHC	Major histocompatability complex
mM	Millimolar
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide
Mu	Murine

MuLV	Murine leukaemia virus
ND	Not determined
ng	Nanogram
NK	Natural killer cell
NO	Nitric oxide
OD	Optical density
p	Probability
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline Tween
PCR	Polymerase chain reaction
PE	Phycoerythrin
pfu	Plaque forming unit
pg	Picogram
r	Recombinant
R	Receptor
R-MuLV	Rauscher murine leukemia virus
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SFFV	Spleen focus forming virus
TcR	T cell receptor
TdR	Thymidine deoxyribose
Th	T helper
TNF	Tumour necrosis factor
U	Unit
UV	Ultraviolet

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Chapter 1

Introduction and Literature Review

1.1 THE IMMUNE RESPONSE - AN OVERVIEW

The immune response toward a pathogen occurs at two levels - an innate and an adaptive response. The innate immune response is rapid, antigen independent and generally not restricted by MHC haplotype. It is mediated by macrophages, monocytes and natural killer (NK) cells as well as non-cellular factors that include interferon (IFN) and complement (Janeway Jr, 1989) The aim of the innate immune response is to limit early pathogen replication during an infection as well as to promote the development of an adaptive immune response (Romagnani, 1992).

The adaptive immune response is characterised by the activation and differentiation of T and B lymphocytes to effector function. It takes several days longer to generate than the innate response, is antigen dependent and restricted by MHC haplotype. A wide range of host adaptive immune responses may be generated following infection with a pathogen or immunisation with an antigen. These include the generation of humoral and cell mediated immune (CMI) responses. The former response is associated with the production of antibodies by B cells while the latter is characterised by the generation of delayed type hypersensitivity (DTH), activated macrophages, cytotoxic $CD8^+$ T cells and the production of IgG 2a antibody (Mosmann and Coffman, 1989). The type and magnitude of the response is tightly regulated and coordinated by the production of cytokines and cell surface molecules.

1.2 THE ADAPTIVE IMMUNE RESPONSE

1.2.1 Antigen recognition

The central problem faced by the immune system is to distinguish between proteins (antigens) synthesised by normal host cells (self) and proteins produced by infectious agents (non-self). The innate immune response is activated largely independently of antigen. It is induced by alternative stimuli including virus infection, microbial products such as bacterial LPS and altered expression of MHC class I molecules on the surface of cells (Müller-Eberhard, 1989). In contrast, the adaptive immune response is driven by recognition of non-self antigens by lymphocytes. Lymphocytes

recognise antigen through clonally restricted antigen receptors expressed on their cell surface. B cells recognise 3 dimensional shapes of proteins in their native state through cell associated antibody (Reth, 1992). In contrast, antigen recognition by most T cells occurs through the α/β chain heterodimeric T cell receptor. These receptors recognise small, proteolytic fragments of protein antigens that are presented at the target cell surface in association with either class I or class II MHC molecules (Matis, 1990). Dependence on antigen ensures that the adaptive immune response is specific and that it is down regulated once the antigen has been cleared.

1.2.2 Antigen presentation to T cells

The recognition of antigen by T lymphocytes is central to the generation and mediation of adaptive immune responses. As described above, T cells recognise antigen derived peptides presented in association with MHC molecules. Specific T cell subsets recognise antigen bound to particular classes of MHC molecule. T cells expressing CD8 recognise cells presenting antigen in association with MHC class I molecules, while $CD4^+$ T cells recognise peptides presented by MHC class II molecules. The source of peptides presented by each class of MHC molecule determines the function of the T cell subsets. Class I MHC molecules present peptides derived from proteins synthesised by the cell or present in the cytoplasm (reviewed in Braciale *et al.*, 1987; Jardetzky *et al.*, 1991). Virtually all nucleated cells express, or can be induced to express, MHC class I molecules. Activated $CD8^+$ T cells can, therefore, detect cells producing non-self proteins eg. virus infected cells, or those expressing mutated self proteins eg. tumour cells (Townsend *et al.*, 1985; Townsend *et al.*, 1986; Fremont *et al.*, 1992; Wallny *et al.*, 1992; reviewed in Engelhard, 1994). In contrast, peptides presented by MHC class II molecules are generally derived from proteins produced outside the antigen presenting cell (Braciale *et al.*, 1987). These proteins are internalised via endocytosis or by antibody mediated uptake of antigen and are processed in lysosomal or endosomal compartments (Cresswell, 1994). It is within these compartments that peptides derived from exogenous antigen associate with MHC class II molecules before being transported to the cell surface. The expression of MHC class II molecules is largely restricted to dendritic cells, macrophages and B cells,

although MHC class II expression can be induced on fibroblasts and endothelial cells (Benoist and Mathis, 1990).

1.2.3 T cell activation

T cell activation requires two signalling events (reviewed in Bretscher, 1992). Signal 1 is delivered following ligation of the T cell receptor by antigen presented in association with MHC molecules on an antigen presenting cell (APC). The provision of signal 2 (co-stimulation) is then required to stimulate T cells to produce cytokines, to proliferate and to differentiate to effector function (Linsley and Ledbetter, 1993). Without signal 2, T cells become unresponsive to further stimulation (anergic) or undergo apoptosis (Harding *et al.*, 1992; reviewed in Jenkins, 1992). The T cell ligand-APC receptor pairs that mediate costimulation include CD28 - B7-1/B7-2 (Linsley *et al.*, 1991; Harding *et al.*, 1992; Harding and Allison, 1993), CD40L - CD40 (de Boer *et al.*, 1993), CD2 - LFA-3 (Moingeon *et al.*, 1989), LFA-1 - ICAM-1,-2 (van Seventer *et al.*, 1990), unknown - HSA (Liu *et al.*, 1992) and CD5 - CD72 (Verwilghen *et al.*, 1993). The ligation of CD28 or CTLA-4 by B7-1 or B7-2 appears to be the main co-stimulatory signal to $CD4^+$ and $CD8^+$ T cells. The other above mentioned receptor-ligand events augment the co-stimulatory response provided by this interaction (Linsley *et al.*, 1991; Harding *et al.*, 1992; Azuma *et al.*, 1993; Harding and Allison, 1993; van Gool *et al.*, 1993; reviewed in Croft, 1994). Seder *et al.*, (1994) showed that IL-2 could replace the co-stimulatory activity of the B7-CD28 interaction which suggests that the role of costimulation may be to augment IL-2 production which in turn induces T cell proliferation and further cytokine production.

1.3 REGULATION OF THE IMMUNE RESPONSE

A wide range of immune responses can be generated toward an antigen. A CMI response is often effective in controlling intracellular viruses, bacteria and parasites (Stueckemann *et al.*, 1982; Karupiah and Harris, 1995), while a humoral immune response mediates immunity to extracellular bacteria and some nematode worms (Urban *et al.*, 1992; Gotschlich, 1993). It has been known for some time that humoral and CMI responses can manifest as mutually exclusive phenomena (Parish, 1972). In 1986, Mosmann *et al.*,

(1986b) demonstrated that murine $CD4^+$ T cell clones often expressed mutually exclusive patterns of cytokines which has led to the hypothesis that cytokines are responsible for the reciprocal nature of CMI and humoral immune responses (reviewed in Mosmann and Coffman, 1989).

1.3.1 Cytokine production by T cell clones

Long term murine $CD4^+$ T cell clones can be subdivided into 2 groups based on the cytokines they produce. T helper 1 (Th1) clones produce IFN-γ, interleukin (IL) -2 and lymphotoxin while T helper 2 (Th2) clones produce IL-4, IL-5, IL-6 and IL-10. Both Th1 and Th2 clones express TNF-α, granulocyte macrophage colony stimulating factor (GM-CSF) and IL-3 (Mosmann *et al.*, 1986b; Mosmann and Coffman, 1989). Some clones express both Th1 and Th2 type cytokines and have been designated Th0 (Firestein *et al.*, 1989; Street *et al.*, 1990). Cloned $CD8^+$ T cells tend to express Th1 cytokines (Fong and Mosmann, 1990), however recent evidence suggests that under certain conditions, $CD8^+$ T cell clones may produce Th2 cytokines (Seder *et al.*, 1992a; Erard *et al.*, 1993; see also Section 1.3.2).

The profile of cytokines produced by each subset of T cells is associated with different immune functions. Adoptive transfer studies showed that Th1 clones supported the generation of DTH while Th2 clones did not (Cher and Mosmann, 1987; Fong and Mosmann, 1989). Both Th1 and Th2 clones can induce antibody production both *in vivo* and *in vitro* (Stevens *et al.*, 1988; Rizzo *et al.*, 1992), however *in vitro* studies indicate that Th2 cells may be more proficient than Th1 cells in this respect (Killar *et al.*, 1987). This is thought to be due to the lack of IL-2 production by some Th1 clones (Killar *et al.*, 1987; Coffman *et al.*, 1988). In addition, Th1 and Th2 clones induce B cells to produce different antibody isotypes. Both clones induce the production of IgM and IgG3 while only Th1 cells augment IgG2a and only Th2 clones promote IgG1 and IgE production (Stevens *et al.*, 1988).

The activity of Th1 and Th2 clones is dependent on the cytokines they produce. Whilst a number of studies have shown that antibody production requires cell contact between activated T cells and B cells

(reviewed in Parker, 1993), this interaction does not directly influence the isotype of antibody produced by stimulated B cells. Rather, the isotype is determined by the cytokines produced by the T cell clone (Hodgkin *et al.*, 1990; Noelle *et al.*, 1991). Similarly, the generation of DTH by antigen specific T cell clones is largely due to IFN-γ produced by Th1 clones (Cher and Mosmann, 1987; Fong and Mosmann, 1989).

It has been conventional to refer to $CD4^+$ T cell clones as T helper cells and to the cytokines they produce as either Th2 or Th1. This nomenclature is somewhat of a misnomer as $CD4^+$ T cells have many roles other than providing the stimulus or 'help' for B cells to proliferate and differentiate to antibody producing cells. In addition, studies over the last 5 years have clearly shown that the Th1-Th2 model developed using *in vitro* generated, long-term T cell clones represents a vast oversimplification of the immune response generated *in vivo*. Cytokines are produced by cells other than $CD4^+$ T cells. These include macrophages (Ehlers *et al.*, 1994) NK cells (Cuturi *et al.*, 1989; Beckerman *et al.*, 1993) $CD8^+$ T cells (Morris *et al.*, 1982; Fong and Mosmann, 1990) and mast cells (Plaut *et al.*, 1989), which can also contribute to the generation and mediation of an effective immune response. To take into account the differing origins of cytokines, a new nomenclature has been proposed in which the Th1 and Th2 terminology described above, has been replaced with type 1 and type 2 cytokines, respectively (Cox and Liew, 1992; Clerici and Shearer, 1994). This system has the advantage of being based on cytokine function rather than on the cell type which produces the factor. In addition, the criteria have been widened to encompass cytokines not produced by $CD4^+$ T cells. For example, IL-12, produced by macrophages and B cells (Cuturi *et al.*, 1989; Kobayashi *et al.*, 1989; D'Andrea *et al.*, 1992) is referred to as a type 1 cytokine since it plays an integral role in promoting development of cells which produce other type 1 cytokines. In the remainder of this thesis, Th1 and Th2 nomenclature will be replaced with type 1 and type 2 as described above, except when referring to $CD4^+$ T cell clones.

1.3.2 The generation of adaptive immune responses

A number of factors determine how the immune response develops following challenge with an antigen. It is clear from *in vitro* and *in vivo*

studies that the cytokines present during the initial stages of T cell activation affect the differentiation of $CD4^+$ and $CD8^+$ T cells (O'Garra and Murphy, 1994). The role of cytokines in the generation of adaptive immune responses are illustrated in Figure 1.1. Other factors also influence the type of immune response generated. These include the route of administration of antigen, the nature of the antigen itself, the type of antigen presenting cell and the dose of antigen (Parish and Liew, 1972; Gajewski *et al.*, 1991b; Bretscher *et al.*, 1992; reviewed in Parish, 1972; Seder and Paul, 1994).

The cytokine milieu present at early stages of $CD4^+$ T cell stimulation markedly influences the development of these cells to secrete different profiles of cytokines (Le Gros *et al.*, 1990; Swain *et al.*, 1990; Gajewski *et al.*, 1991a). The type 1 factors IL-12 and IFN-γ promote the differentiation of stimulated naive T cells into type 1 cells (Macatonia *et al.*, 1993b; Manetti *et al.*, 1993; Seder *et al.*, 1993; Schmitz *et al.*, 1994). Several studies indicate that IL-12 acts directly on naive T cells (Manetti *et al.*, 1993; Seder *et al.*, 1993), however Macatonia *et al.*, (1993b) showed that IFN-γ induced by IL-12 may also may stimulate differentiation of $CD4^+$ T cells to a type 1 phenotype *in vitro*. IL-12 also influences cytokine expression by $CD4^+$ T cells *in vivo*. Endogenous IL-12 production is required for the generation of type 1 responses against intracellular parasites (Sypek *et al.*, 1993) while administration of IL-12 promotes type 1 responses during intracellular parasite infestation and viral infection (Heinzel *et al.*, 1993; Orange *et al.*, 1994). IL-12 promotes CMI responses by directly acting on $CD4^+$ T cells to generate type 1 cells (Manetti *et al.*, 1993) but also by activating NK cells and $CD8^+$ CTL (Kobayashi *et al.*, 1989; Gately *et al.*, 1993; Chouaib *et al.*, 1994).

IFN-γ is also involved in the generation of type 1 responses. Addition of IFN-γ to cultures of naive T cells inhibits the development of IL-4 producing cells and yields $CD4^+$ T cells that express IFN-γ upon restimulation (Gajewski *et al.*, 1991a; Maggi *et al.*, 1992). The role of IFN-γ in directly promoting the development of type 1 cells is less clear. Studies by Seder *et al.*, (1992b) suggest that IFN-γ does not promote the generation of type 1 cells. In contrast, Swain *et al.*, (1990) found that high levels of

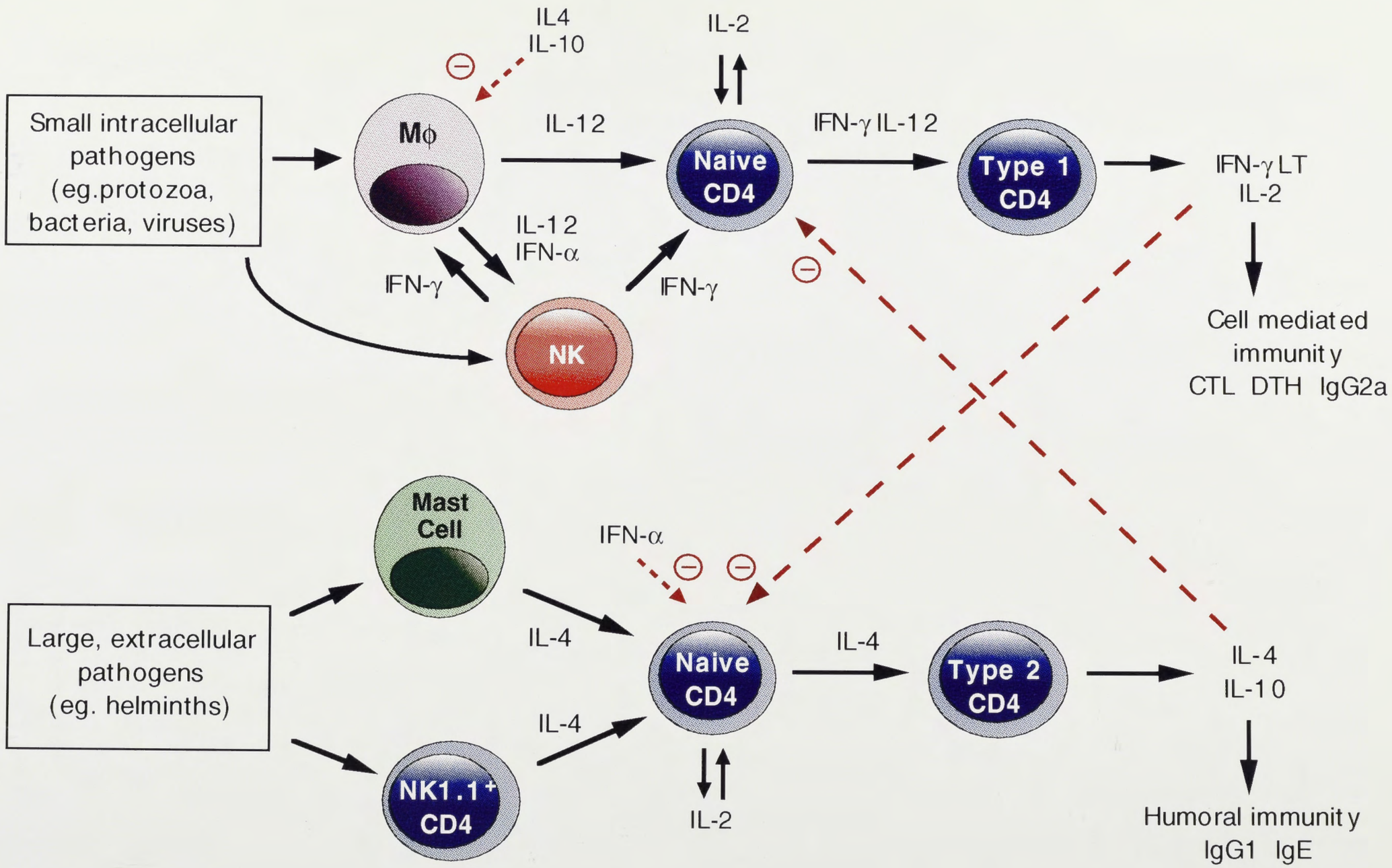

Small intracellular pathogens (eg.protozoa, bacteria, viruses)
Mϕ
IL4
IL-10
IL-12
IL-2
Naive CD4
IFN-γ IL-12
Type 1 CD4
IFN-γ LT
IL-2
Cell mediated immunity
CTL DTH IgG2a
IL-12
IFN-α
IFN-γ
IFN-γ
NK
Large, extracellular pathogens (eg. helminths)
Mast Cell
IFN-α
IL-4
IL-4
NK1.1+ CD4
Naive CD4
IL-4
Type 2 CD4
IL-4
IL-10
IL-2
Humoral immunity
IgG1 IgE

Figure 1.1 ***Role of cytokines in the generation of adaptive immune responses***

Activated $CD4^+$ T cells are integral to the regulation of adaptive immune responses. These cells mediate this regulation via the production of cytokines. However, naive T cells predominantly produce only IL-2. The cytokines present during activation of $CD4^+$ T cells markedly influences cytokines produced by $CD4^+$ T cells.

Cytokines produced early in an immune response by activated macrophages and NK cells such as IL-12 and IFN-γ act upon naive $CD4^+$ T cells to induce their differentiation into cells which express type 1 cytokines (IFN-γ, LT and IL-2). Activated $CD4^+$ T cells which express type 1 cytokines promote the development of cell mediated immune (CMI) responses such as the generation of cytotoxic T lymphocytes (CTL), delayed type hypersensitivity (DTH) and the production of IgG2a antibody. Stimuli such as virus infection or factors produced by intracellular bacteria and parasites lead to the activation of macrophages and NK cells and the generation of CMI responses.

Early production of IL-4 during an immune response promotes the differentiation of naive $CD4^+$ T cells into cells which express type 2 cytokines (IL-4, IL-10). The production of these factors is associated with the production of a humoral immune response characterised by the production of antibodies of the IgG1 and IgE subclasses. The source of early IL-4 production is unclear however there is evidence to suggest that it may come from mast cells and a rare population of $CD4^+$ $NK1.1^+$ T cells. Stimuli such as helminth infestations are associated with the production of type 2 cytokines.

Type 1 cytokines have been shown to mutually antagonise the production of type 2 cytokines. Similarly type 2 factors impair production of type 1 cytokines. The balance of early production of type 1 or type 2 cytokines is integral to the generation of CMI or humoral immune responses. The antagonistic effects of each cytokine type may act upon naive $CD4^+$ T cells or other cells involved in the generation of adaptive immune responses. In this figure, black arrows indicate positive signals and red arrows indicate negative signals.

Abbreviations: IL - interleukin; IFN - interferon, NK - natural killer, Mϕ - macrophage; LT - lymphotoxin

IFN-γ promotes type 1 cell development in the presence of low levels of IL-4. Furthermore, as described above, neutralisation of IFN-γ in the presence of IL-12 inhibited type 1 cell generation *in vitro* (Macatonia *et al.*, 1993b). The reason for these differing results is unknown, however they may be due to the different strains of mice used or differences in culture conditions (Seder and Paul, 1994). IFN-γ has also been shown to promote the development of type 1 cells *in vivo*. Mice treated with anti-IFN-γ mAb fail to generate type 1 responses following challenge with the protozoan *Leishmania major* (Belosevic *et al.*, 1989; Scott, 1991). Furthermore, the ability of IL-12 to induce a type 1 response is dependent on IFN-γ in a number of *in vivo* disease models (Heinzel *et al.*, 1993; Gazzinelli *et al.*, 1994b; Oswald *et al.*, 1994a).

Several studies have shown that the presence of IFN-α can promote the generation of Th1 $CD4^+$ T cell clones (Parronchi *et al.*, 1992; Brinkmann *et al.*, 1993). The mechanism by which IFN-α promotes the generation of type 1 cells is unclear, however several studies suggest that IFN-α may act by inhibiting IL-4 transcription (Finkelman *et al.*, 1991) and promote IFN-γ production by NK cells (Biron *et al.*, 1984; Kobayashi *et al.*, 1989) which may further augment the development of type 1 cells (Varkila *et al.*, 1993).

Although IL-2 is produced by most Th1 T cell clones (Mosmann *et al.*, 1986b; Street *et al.*, 1990), the factor has little effect on the development of cytokine production by naive $CD4^+$ T cells (Swain, 1991). Rather, IL-2 is thought to act as a growth factor for stimulated T cells (Seder *et al.*, 1994).

IL-4 is a critical factor in the commitment of $CD4^+$ T cells to the expression of a type 2 phenotype both *in vitro* and *in vivo*. Naive $CD4^+$ T cells are poor producers of IL-4 *in vitro*, however, the presence of IL-4 during initial stimulation of these cells promotes the expression of IL-4 upon restimulation (Le Gros *et al.*, 1990; Swain *et al.*, 1990). This effect is mediated by a direct action of IL-4 on T cells and occurs independently of the type of APC used (Hsieh *et al.*, 1992). Furthermore, the presence of the type 1 factors IFN-γ or IL-12 does not diminish the ability of IL-4 to promote the generation of type 2 cells (Seder *et al.*, 1992b; Scmitt *et al.*, 1994). IL-4 also plays a major role in the development of type 2 responses

in vivo (Sadick *et al.*, 1990; Chatelain *et al.*, 1992; Kopf *et al.*, 1993). The generation of type 2 cells is also augmented by IL-10. *In vitro* studies suggest that IL-10 does not directly promote the generation of type 2 cells, rather it inhibits the development of type 1 cells (Hsieh *et al.*, 1992; Macatonia *et al.*, 1993a). The mechanism by which IL-10 inhibits type 1 cell development is unclear, however IL-10 is known to act directly on macrophages which in turn prevent cytokine production by Th1 clones (Fiorentino *et al.*, 1991). Thus it is possible that IL-10 may act indirectly via APC to block the generation of type 1 cells.

The differentiation of naive $CD8^+$ T cells is also dependent on cytokines present during primary stimulation. The generation of classical MHC class I restricted, lytic, IFN-γ producing $CD8^+$ T cells *in vitro* is dependent on IL-2 (Erard *et al.*, 1985). IFN-γ, in the presence of IL-2, may also influence CTL generation (Maraskovsky *et al.*, 1989). Other factors that promote the generation of $CD8^+$ CTL include IL-1 and TNF-α (Vink *et al.*, 1990), IL-6 (Quentmeier *et al.*, 1992; Kopf *et al.*, 1994), IL-7 (Kos and Müllbacher, 1992) and IL-12 (Gately *et al.*, 1993; Chouaib *et al.*, 1994). In marked contrast, naive $CD8^+$ T cells cultured in the presence of IL-4 lose the ability to express CD8, are not cytolytic and do not produce IFN-γ (Erard *et al.*, 1993; Tanaka *et al.*, 1993). These cells express IL-4, IL-5 and IL-6 and are capable of helping B cells produce antibody (Erard *et al.*, 1993; Tanaka *et al.*, 1993; Cronin *et al.*, 1995).

The powerful influence of cytokines on the generation of particular immune responses raises the question of what stimulates these factors to be produced. The source of IL-4 that leads to priming of T cell IL-4 production *in vivo* is unclear. *In vitro* studies indicate that IL-4 is required to induce T cells to produce IL-4 (Le Gros *et al.*, 1990; Swain *et al.*, 1990). There is some speculation that the initial source of IL-4 is non-T cells such as activated mast cells or basophils (Plaut *et al.*, 1989; Ben-Sasson *et al.*, 1990; Romagnani, 1992). However, a recent study using IL-4 deficient mice reconstituted with naive $CD4^+$ T cells from congenic, normal mice, clearly demonstrated that these cells are capable of generating sufficient IL-4 for the induction of type 2 responses *in vivo* (Schmitz *et al.*, 1994). It is therefore interesting to note that a rare

population of $CD4^+$ T cells which express the marker NK 1.1 have been found to express IL-4 independently of prior exposure to IL-4 (Yoshimoto and Paul, 1994).

The early production of IL-12 and IFN-α may promote the production of type 1 responses. In the absence of IL-4, IL-12 induces the differentiation of T cells into cells that secrete IFN-γ (Seder *et al.*, 1993) while IFN-α inhibits IL-4 production (Finkelman *et al.*, 1991). Furthermore, IL-12 and IFN-α activate NK cells and stimulate IFN-γ production (Biron *et al.*, 1984; Kobayashi *et al.*, 1989) which may further augment the development of type 1 cells (Varkila *et al.*, 1993). Macrophages produce IL-12 and IFN-α following stimulation with viruses (Samuel, 1991; Coutelier *et al.*, 1995) parasites (Sypek *et al.*, 1993; Gazzinelli *et al.*, 1994b) and bacterial antigens (D'Andrea *et al.*, 1992). These observations suggest that NK cells and macrophages are important factors in the induction of type 1 cytokines and CMI responses (Romagnani, 1992).

The dose of antigen also plays a role in determining the type of immune response which is generated. Bretscher *et al.*, (1992) demonstrated that BALB/c mice infected with low doses of *L. major* generate an effective and long lasting CMI response. In marked contrast, when these mice were given much higher doses of *L. major* an ineffective humoral response resulted. This extends similar findings of (Parish and Liew, 1972) with inert immunogenic proteins to infectious, replicating organisms. The potential for manipulation of the class of immune response generated by the amount of antigen given clearly has important implications for the rational design of vaccines.

The type of APC involved in the generation of an immune response may also influence the development of type 1 or type 2 cells. Gajewski *et al.*, (1991b) showed that T cells prepared from the same lymph nodes could develop into type 1 or type 2 cells depending on the APC used in the culture system. Stimulation of lymph node cells in the presence of B cells promoted the generation of type 2 $CD4^+$ T cells, while macrophages or dendritic cells were required for maximal proliferation of type 1 $CD4^+$ T cells. Although co-factors responsible for these differences were not identified, they were shown to be produced by the APCs themselves.

Other studies have shown that such effects may be mediated by cytokines. IL-12 produced by macrophages (Hsieh *et al.*, 1993), or the action of IL-10 on macrophages or dendritic cells (Hsieh *et al.*, 1992) can influence the phenotype of $CD4^+$ T cells generated *in vitro*. In contrast, IL-4 directs the development of type 2 cells independently of the APC used (Seder *et al.*, 1992b).

1.3.3 Cross-regulation of the immune response

As described above in Section 1.3.2, type 1 and type 2 cytokines regulate the expression of the opposite subset during the induction of an immune response. Fully differentiated $CD4^+$ T cells can also exert such cross regulation. Early studies of T cell clones showed that IFN-γ produced by Th1 clones suppressed the proliferation of Th2 clones but not their production of cytokines such as IL-4 (Fernandez-Botran *et al.*, 1988; Gajewski and Fitch, 1988). In contrast, the type 2 cytokine IL-10, inhibits IFN-γ production by Th1 clones (Fiorentino *et al.*, 1991). This mechanism of *in vitro* cross-regulation by Th1 and Th2 clones may underlie immune regulation *in vivo* and therefore explain the exclusive generation of DTH or antibody responses described by (Parish and Liew, 1972). Subsequent investigations of cytokine cross-regulation *in vivo* found that neutralising anti-IL-4 or anti-IFN-γ mAb did not alter the immune response in mice with established *L. major* infection (Belosevic *et al.*, 1989; Sadick *et al.*, 1990; Chatelain *et al.*, 1992). Further studies demonstrated that CMI against *L. major* can be markedly downregulated by *in vivo* therapy with IL-4 and IL-10 (Powrie and Coffman, 1993b). In contrast, administration of IL-12 to mice with an established humoral (type 2) immune response did not alter expression of IL-4 (Wang *et al.*, 1994). This phenomenon may be due to impaired IL-12 induced signalling in cells expressing type 2 factors (Szabo *et al.*, 1995). These findings suggest that only in some situations cytokines may prove useful as therapeutic agents in the treatment of long term diseases by converting an ineffectual immune response into one that mediates protection.

Type 1 and type 2 cytokines also act on non-$CD4^+$ T cells and modulate their function. For example, IL-4 inhibits macrophage cytotoxicity by downregulating expression of nitric oxide (Bogdan *et al.*, 1994), while IFN-γ

activates macrophages and induces nitric oxide expression (Ding *et al.*, 1988). The role of cytokines in cross-regulating immune effector mechanisms is discussed in later sections.

1.3.4 Cytokine responses in infectious disease

The success of an immune response against an infectious organism is dependent on the activation of an appropriate set of immune effector responses. The inability to control a pathogen often results from the generation of an inappropriate immune response rather than a failure to generate an immune response at all (Bretscher *et al.*, 1992; Powrie and Coffman, 1993a). A number of models of parasite infestation and bacterial infection exhibit marked polarisation of cytokine expression and type of immune response generated (Sher *et al.*, 1992). Perhaps the model that best illustrates the marked exclusivity of type 1 and type 2 cytokine production *in vivo* is *L. major* infection of mice (reviewed in Reed and Scott, 1993). Infection of inbred mouse strains yields at least two distinct disease patterns. C3H/He or C57BL/6 mice produce a strong CMI response toward *L. major* that effectively clears the parasite. $CD4^+$ T cells from these mice produce high levels of the type 1 cytokines IFN-γ and IL-2 and low levels of type 2 factors such as IL-4 (Heinzel *et al.*, 1989; Heinzel *et al.*, 1991). In contrast, BALB/c mice produce a predominantly IgG1 antibody response to infection with *L. major* and weak DTH responses which do not control infection. These mice eventually die from disseminated leishmaniasis. $CD4^+$ T cells from infected BALB/c mice predominantly produce the type 2 factors IL-4, IL-5 and IL-10 (Heinzel *et al.*, 1989; Heinzel *et al.*, 1991). Evidence that $CD4^+$ T cells expressing type 1 factors mediate resistance to *L. major* was provided in adoptive transfer studies using T cell clones expressing different patterns of cytokines (Scott *et al.*, 1988).

Cytokine expression during viral infections also influences the development of an effective antiviral immune response. Resistance to viral infections with lymphocytic choriomeningitis virus (LCMV), ectromelia and vaccinia virus is dependent on the generation of antiviral CTL and the production of type 1 cytokines such as IFN-γ (Klavinskis *et al.*, 1989; Leist *et al.*, 1989; Ruby and Ramshaw, 1991; Karupiah *et al.*, 1993a;

Carpenter *et al.*, 1994). In sharp contrast, expression of IL-4 by recombinant vaccinia virus markedly impairs the generation of CTL and significantly delays viral clearance (Sharma *et al.*, 1996). Furthermore, expression of type 1 cytokines IL-2, IFN-γ and IL-12 are also downregulated.

There are examples of immune responses where the pattern of cytokine expression is not highly polarised. For example, analysis of cytokine mRNA and protein production by T cells from the lungs and lymph nodes of mice infected with influenza virus demonstrate that both type 1 and type 2 factors are expressed during infection (Carding *et al.*, 1993; Sarawar and Doherty, 1994). These data suggest that the preferential generation of a type 1 response is not essential to the clearance of some viral infections.

A strong CMI response (characterised by expression of type 1 cytokines) is thought to be important in the control of HIV infection (Clerici *et al.*, 1992; Salk *et al.*, 1993). Recently, Clerici and Shearer, (1993) proposed that the progression of HIV infected individuals to AIDS was due to the gradual loss of $CD4^+$ T cells which produce type 1 cytokines and increased production of Th2 cytokines. Other investigations of cytokine expression during HIV infection have tended not to support this type 1-type 2 switch model. Using *in situ* hybridisation to detect cytokine expression in the lymph nodes of HIV infected individuals, Emile *et al.*, (1994) found no evidence for elevated IL-4 expression at any stage of disease, while expression of IL-2 and IFN-γ were markedly elevated. In a limited study, Graziosi *et al.*, (1994) showed no increase in lymph node mRNA expression IL-4 mRNA. Furthermore, Maggi *et al.*, (1994) failed to confirm that cytokine production by peripheral blood mononuclear cells to recall antigens switched from type 1 to a type 2 cytokine production pattern however, an increase in cells with a Th0 phenotype was observed. In a reappraisal of their original model, Clerici and Shearer, (1994) suggested that these observations which are inconsistent with their original hypothesis may reflect differences in experimental design and the material used.

1.4 CELLULAR ANTIVIRAL EFFECTOR MECHANISMS

1.4.1 NK cells

Natural killer (NK) cells are large, granular, $CD2^+$, $CD3^-$, TcR^-, Ig^- lymphocytes which lyse a number of virus infected and tumour cells without prior stimulation (reviewed in Trinchieri, 1989). NK cells mediate antiviral effects *in vivo* by lysing infected cells and producing antiviral cytokines including IFN-γ and TNF-α (Trinchieri, 1989). Natural killer cells also produce GM-CSF and IL-3 (Perussia, 1991) Together, these factors have roles in the activation of other immune cells, such as macrophages (Bancroft *et al.*, 1987) to effector function and may promote the generation an adaptive immune response (Romagnani, 1992; Scharton and Scott, 1993).

The nature of the receptors used by NK cells to recognise tumour cells or cells infected with virus is unclear. A number of investigators have demonstrated that expression of MHC class I molecules protects tumour cells from lysis by NK cells (Kärre *et al.*, 1986; Liao *et al.*, 1991). Furthermore, Storkus *et al.*, (1991) showed that mutations in the peptide binding groove of MHC class I molecules could render tumour cells susceptible to lysis by NK cells. These observations suggest that NK cells recognise MHC class I molecules associated with peptides derived from self proteins and that loss of MHC class I expression or displacement of self peptides with mutant self or viral peptides could render such a cell susceptible to NK cell mediated lysis (Raulet, 1992).

Viral infection can enhance NK cell cytotoxic activity and stimulated their proliferation of these cells. These effects can be induced by IFN-α and IFN-β which is produced by infected cells and activated macrophages (Biron and Welsh, 1982; Biron *et al.*, 1983; Biron *et al.*, 1984; Bigda *et al.*, 1994). In addition, viral peptides and recognition of virus infected target cells activate NK cells (Welsh, 1986). Other cytokines, such as IL-12 (Kobayashi *et al.*, 1989) and IL-2 (Biron, 1990) activate NK cells and induce their proliferation, while factors such as IL-10 and TGF-β1 inhibit NK cell function (Su *et al.*, 1991).

The early activity of NK cells during virus infection permits them to act as a front line defence mechanism well before the generation of an adaptive immune response. Moreover, the rapid production of cytokines by NK cells may promote the generation of effective innate and adaptive immune responses.

1.4.2 Macrophages

Resident tissue macrophages and blood monocytes (referred to here as macrophages) are bone marrow derived mononuclear phagocytic cells. These cells are major anti-viral effectors of both the innate and adaptive immune responses and are integral to the induction and regulation of the latter (Blanden, 1982). During virus infections, macrophages mediate antiviral activity by direct actions on virus infected cells, or by promoting or down regulating various immune functions (Morohan *et al.*, 1985).

Activated macrophages can directly limit virus replication by producing antiviral cytokines such as IFN-β and TNF-α (Goldfield and Maniatis, 1989) virucidal reactive oxygen intermediates (Chase and Klebanoff, 1992), reactive nitrogen intermediates (RNI; Croen, 1993; Karupiah *et al.*, 1993b); see also Section 1.5.4). Macrophages also phagocytose viral particles (reviewed in Kaufmann and Reddehase, 1989) and mediate antibody dependent cellular cytotoxicity against virus infected cells (see Section 1.5.6).

Macrophages promote the generation of antiviral immune responses in a number of ways. Their production of cytokines such as IFN-α and IL-12 enhances NK cell cytotoxicity and cytokine production (Biron *et al.*, 1984; Kobayashi *et al.*, 1989) and promotes generation of a CMI response (Finkelman *et al.*, 1991; D'Andrea *et al.*, 1992; Manetti *et al.*, 1993). Macrophages also present phagocytosed extracellular antigens to $CD4^+$ T cells in association with MHC class II molecules, thereby initiating antiviral immune responses. Following activation with IFN-γ, macrophage expression of MHC class II molecules is upregulated (Wong *et al.*, 1983) which, in turn, may increase antigen presentation to $CD4^+$ T lymphocytes.

Activated macrophages can also modulate immune responses. Production of high levels of nitric oxide by macrophages can suppress T cell proliferation and antibody production (Albina *et al.*, 1991; Al-Ramadi *et al.*, 1992). Other factors produced by macrophages, such as prostaglandins or steroid hormones impair B cell proliferation (Albina *et al.*, 1991) or alter the production of cytokines by T cells (Daynes *et al.*, 1991) respectively.

The stimulation of macrophages to effector function is a multistep process requiring several signals. These signals can be delivered by cytokines, in particular IFN-γ and TNF-α. IFN-γ can provide the first signal to prime macrophages (Nathan *et al.*, 1983; Pace *et al.*, 1983). Primed macrophages do not exhibit cytotoxic activity and require a further signal to become activated and cytotoxic (Pace *et al.*, 1983). Following priming with IFN-γ, macrophages express TNF-α receptors (Ruggiero *et al.*, 1986) and TNF-α which stimulates the macrophage further (Philip and Epstein, 1986). Bacterial LPS can also prime macrophages to produce TNF-α and respond to secondary stimuli (such as TNF-α) and has been used as a convenient agent for macrophage stimulation. However, where bacteria are absent, as in viral infections, other mechanisms of macrophage activation occur. Activated T cells can provide the signals necessary for macrophage activation (reviewed in Stout, 1993), for example IFN-γ and TNF-α produced by activated type 1 T cells. IFN-γ primed macrophages may also be activated by antigen specific cell surface interactions with activated, but not resting, type 1 or type 2 T cells (Stout and Suttles, 1993; Tao and Stout, 1993). Recently Tian *et al.*, (1995), demonstrated that signalling occurs via ligation of CD40 and ICAM-1 (on the macrophage) by CD40L and LFA-1 (on the T cell). Fixed Th2 cells may activate IFN-γ primed macrophages which indicates that cell surface interactions between type 1 and type 2 T cells and macrophages may be similar (Stout and Suttles, 1993).

Macrophage activity is down-regulated by the cytokines IL-4, IL-10 and TGF-β. These cytokines inhibit macrophage effector functions including NO synthesis (Vodovotz *et al.*, 1993; Bogdan *et al.*, 1994) and the production of antiviral cytokines such as TNF-α (Bogdan *et al.*, 1991; Gautam *et al.*, 1992; Hausmann *et al.*, 1994). Furthermore, IL-4, IL-10 or TGF-β, either alone or together, down-regulate macrophage cytotoxicity

(Oswald *et al.*, 1992). In addition, IL-4 inhibits the ability of macrophage produced IL-12 to induce type 1 $CD4^+$ T cell development (Hsieh *et al.*, 1993; Schmitz *et al.*, 1994), while IL-10 also suppresses the ability of macrophages to generate type 1 cells (Hsieh *et al.*, 1992).

1.4.3 Cytotoxic T cells

The recognition and killing of virus infected cells by antigen specific, activated, cytotoxic T cells (CTL) is a major antiviral effector mechanism of the adaptive immune response (Blanden, 1974). Both $CD8^+$ and $CD4^+$ T cells exhibit cytotoxicity (Zinkernagel and Althage, 1977; Lukacher *et al.*, 1984; Lukacher *et al.*, 1985) however MHC class I restricted ($CD8^+$) CTL are thought to constitute the main antiviral CTL in most viral infections (Zinkernagel and Doherty, 1979).

The requirement of non-cytolytic $CD4^+$ T helper cells for the generation of antiviral $CD8^+$ CTL is variable. *In vitro* studies indicate that $CD4^+$ T cells augment the development of $CD8^+$ CTL responses to ectromelia, influenza, Sendai virus and vesicular stomatitis virus (Pang *et al.*, 1976; Ashman and Müllbacher, 1979; Ciavarra, 1990). $CD4^+$ T cells promote the generation of antiviral $CD8^+$ CTL by providing cytokines active in CTL development (see Section 1.3.2.). $CD4^+$ T cells may also be required for the generation of optimal $CD8^+$ antiviral CTL responses *in vivo*, during infection with Sindbis virus (Kast *et al.*, 1986) or mouse hepatitis virus (Williamson and Stohlman, 1990). In contrast, a number of studies have shown that mice lacking $CD4^+$ T cells develop effective antiviral $CD8^+$ CTL against vaccinia (Buller *et al.*, 1987a), influenza (Allan *et al.*, 1990), LCMV (Rahemtulla *et al.*, 1991) or herpes simplex virus-1 (HSV-1; Nash *et al.*, 1987). These experiments suggest that $CD4^+$ T cells are not essential for the generation of $CD8^+$ CTL, however they do not preclude a role for $CD4^+$ T cells in normal mice. It seems likely that $CD4^+$ T cells play a role in the generation of $CD8^+$ CTL, given that $CD8^+$ T cell development is influenced by cytokines such as IL-2, IFN-γ, IL-4 and IL-6 which are produced by activated $CD4^+$ T cells (see Section 1.3.2).

Once activated, CTL do not require co-stimulation by target cells to mediate lysis (Azuma *et al.*, 1993; Harding and Allison, 1993). Lysis by activated

CTL is a sequential process beginning with their adhesion to the virus infected target cell. This is followed by T cell receptor ligation (recognition) by antigen associated with MHC on the target cell surface (reviewed in Berke, 1994). Following recognition, a 'lethal hit' is delivered to the target cell which leads to its death. The CTL then dissociates from the target cell and may carry out several more rounds of target cell lysis.

The mechanism by which CTL lyse virus infected cells has been a topic of debate for many years. Two main models have been developed to explain CTL mediated lysis. It has been proposed that lysis of target cell membranes occurs via secreted pore-forming proteins such as perforin (analogous to complement proteins) as well as proteases (Squier and Cohen, 1994). Alternatively, CTL mediated lysis may be due to the triggering of apoptosis in target cells by ligation of the Fas molecule on the surface of target cells (Rouvier *et al.*, 1993; Stalder *et al.*, 1994). Neither model is sufficient to fully explain the CTL mediated lysis observed in all systems, which implies that two mechanisms of lysis may be acting (Berke, 1994).

Following recognition of target cells, CTL also produce cytokines such as IFN-γ and TNF-α (Morris *et al.*, 1982; Fong and Mosmann, 1990; Carpenter *et al.*, 1994). The antiviral activity of these factors, may directly limit virus replication within infected cells and also protect nearby cells from infection. Furthermore, IFN-γ induces upregulation of MHC class I expression, which may enhance lysis of target cells (Bukowski and Welsh, 1985). The importance of antiviral cytokines in CTL-dependent immunity to viral infection is illustrated by studies of immunity to vaccinia virus. Clearance of vaccinia virus is dependent on antiviral $CD8^+$ T cells (Ruby and Ramshaw, 1991). The generation of antiviral CTL was found to be unaffected by neutralisation of IFN-γ *in vivo* using mAb, however resolution of infection was markedly impaired. These results indicate that antiviral CTL limit vaccinia virus replication by focussing the expression of antiviral cytokines at the site of virus replication (Ruby and Ramshaw, 1991; Ramsay *et al.*, 1993).

1.4.4 B cells

B cells mediate effector function by secreting antibodies. These molecules are globular proteins that bind to 3 dimensional shapes of antigens (epitopes) with high specificity and affinity. Antibodies mediate antiviral effects via a number of mechanisms which are discussed in sections 1.5.5 and 1.5.6.

The T cell-dependent activation of B cells is somewhat analogous to T cell activation, in that 2 signals are required. However, the process of activation is fundamentally different. The first event leading to B cell activation is the specific binding of antigen to surface immunoglobulin (Ig; Signal 1). Following antigen binding, the antigen is endocytosed and proteolyticly degraded in endosomal compartments. Short peptide fragments of the degraded antigen are then expressed on the cell surface in association with MHC class II molecules (Hodgkin and Basten, 1995). The role of antigen binding to Ig in B cell activation has long been a topic for debate (Langman and Cohn, 1991; Moeller *et al.*, 1991). It appears that the strength of intracellular signalling by surface Ig influences subsequent B cell responses. Multivalent antigens such as polysaccharides or high concentrations of monovalent antigens lead to the delivery of strong intracellular signalling by surface Ig which, in turn, leads to T cell independent B cell responses (de Franco *et al.*, 1987; Mongini *et al.*, 1992). On the other hand, low concentrations of monovalent antigens are not sufficient to induce B cell proliferation and the B cell requires further signals to proliferate and produce antibody.

T cell-dependent activation of B cells usually requires stimulatory signals or 'help' from activated $CD4^+$ T cells (reviewed in Parker, 1993). The recognition of peptide associated with MHC class II molecules by the TcR of activated T cells (Signal 2) coupled with costimulation by CD40-CD40L receptor-ligand pairs (B cell-T cell; Armitage *et al.*, 1992; Noelle *et al.*, 1992) induce B cells to proliferate and differentiate. It would appear that ligation of CD40 by CD40L is essential for T cell dependent B cell activation as mice treated with anti-CD40L antibodies fail to generate antibodies toward a number of antigens (Foy *et al.*, 1993). Furthermore, mice lacking CD40 exhibit impaired Ig class switching and germinal centre formation.

Moreover, X-linked hyper-IgM syndrome in humans, which is characterised by defective Ig class switching and no germinal centre formation, is due to mutation in the CD40L gene (Allen *et al.*, 1993). $CD4^+$ T cell clones have been shown to be able to provide costimulation to B cells via cell surface TNF-α, however it is unclear if this mechanism of costimulation is important *in vivo* (Aversa *et al.*, 1993; Macchia *et al.*, 1993). Activated $CD8^+$ T cells that produce IL-4, IL-6 and IL-10 may also provide help for naive B cells (Erard *et al.*, 1993). A recent study has shown that $CD8^+$ T cells which produce type 2 cytokines also express CD40L and provide help for B cells via ligation of CD40 (Cronin *et al.*, 1995).

Following activation, B cell development occurs under the influence of cytokines such as IL-4, IL-5, IL-6, IL-10, IFN-γ and IFN-α, which modulate B cell proliferation, differentiation and the isotype of antibody produced (Snapper and Paul, 1987; Hodgkin *et al.*, 1990; Finkelman *et al.*, 1991).

1.5 NON-CELLULAR ANTIVIRAL EFFECTOR MECHANISMS

1.5.1 Interferon

The IFNs are a family of cytokines produced during viral infections. IFNs exhibit direct antiviral activity *in vitro* via the induction of enzymatic pathways and the synthesis of proteins which limit viral replication and inhibit production of progeny virus (Samuel, 1991). In addition, IFNs promote the generation of antiviral immune responses via the activation and differentiation of immune cells to effector function.

IFNs are classified into 2 separate groups. Type I IFN (IFN-α, IFN-β, IFN-ω) are related proteins that bind to the same receptor. There are over 20 distinct genes encoding IFN-α while there is only one IFN-β and one IFN-ω. Type II IFN (IFN-γ) is genetically and structurally distinct from Type I IFN and acts via a separate receptor (Samuel, 1991). Viral infection can induce the expression of Type I IFN by a wide range of cells including macrophages, T cells, B cells, fibroblasts and epithelial cells. In contrast, viral infection does not directly stimulate IFN-γ production. Rather, IFN-γ is expressed by NK cells following their activation by cytokines or by target

recognition and by T cells after antigenic or mitogenic stimulation (Handa *et al.*, 1983; Sandvig *et al.*, 1987).

IFN-α, IFN-β and IFN-γ induce an antiviral state in uninfected cells which limits virus replication. IFNs mediate their antiviral effects by inducing expression of a number of proteins (reviewed in Staeheli, 1990; Samuel, 1991). IFN induces the expression of 2',5'-oligoadenylate synthetase which catalyses the formation of 2',5'-oligoadenylic acid in the presence of double stranded RNA which is formed during virus replication. The 2',5'-oligoadenylic acid activates an endoribonuclease which degrades both cellular and viral RNA thus inhibiting virus replication (Kerr and Brown, 1978; Baglioni, 1979). As well as 2',5'-oligoadenylate synthetase, IFN induce the expression of the protein kinase P1/eIF-2. When activated by autophosphorylation (in the presence of double stranded RNA) this kinase phosphorylates the α subunit of protein synthesis initiation factor eIF-2 which inhibits protein synthesis by blocking the initiation of translation (Samuel, 1991). The induction of these enzymes and their effects on virus growth are dependent on the type of cell affected and the nature of the virus (Staeheli, 1990). The antiviral effects of each IFN species are often augmented by interactions with other types of IFN (Fleishmann and Schwarz, 1981) or cytokines such as TNF (Wong and Goeddel, 1986; Wong *et al.*, 1988).

The antiviral effects of IFN *in vivo* may be mediated by direct action on cells, as described above, or by activation of cellular effector functions. IFN-α,-β,-γ are involved in the activation of NK cells (Biron and Welsh, 1982; Biron *et al.*, 1983), while IFN-γ induces macrophage activation (Pace *et al.*, 1983; Ding *et al.*, 1988) and generation of CTL (Maraskovsky *et al.*, 1989). In addition, IFN-α and IFN-γ modulate the isotype of antibody secreted by B cells, inducing the production of IgG2a while suppressing the production of IgE (Snapper and Paul, 1987; Sadick *et al.*, 1990; Finkelman *et al.*, 1991).

IFNs have marked effects on the regulation of MHC expression. IFN-α, IFN-β and IFN-γ all induce MHC class I expression but only IFN-γ promotes MHC class II expression (Wong *et al.*, 1983; Bukowski and

Welsh, 1985). Treatment of virus infected cells with IFN renders them more susceptible to lysis by CTL (Bukowski and Welsh, 1985). This may be due to enhanced expression of MHC molecule-associated viral peptides on the surface of infected cells making these cells more recognisable by CTL. Furthermore, the induction of MHC class II by IFN-γ may also increase the presentation of antigen to $CD4^+$ T cells thereby promoting the generation of adaptive immune responses.

The importance of Type I and Type II IFN in resistance to viral infection has been demonstrated using specific antibodies to block IFN activity (Gresser *et al.*, 1976; Klavinskis *et al.*, 1989; Leist *et al.*, 1989; Ruby and Ramshaw, 1991; Karupiah *et al.*, 1993b), genetically altered mice that lack functional IFN or IFN receptor (Dalton *et al.*, 1993; Huang *et al.*, 1993) or administration of purified IFN protein (Hekman and Trapman, 1985; Schijns *et al.*, 1988; Ruprecht *et al.*, 1990). Furthermore, recombinant vaccinia virus encoding murine IFN-γ is markedly attenuated in both normal and athymic nude mice (Kohonen-Corish *et al.*, 1990). The mechanism of virus attenuation is unclear, however the rapid clearance of virus is consistent with direct antiviral activity of IFN-γ or activation of mononuclear phagocytes to antiviral effector function (Karupiah *et al.*, 1993a). It should be noted, however, that the course of infection with influenza virus was unaffected by treatment with antibodies to type I IFN (Gresser *et al.*, 1976), while mice lacking functional IFN-γ are no more sensitive to infection than wild type mice (Graham *et al.*, 1993). These observations indicate that IFN mediate many antiviral effects *in vivo*, although IFN may not be essential for an effective immune responses in all viral infections.

1.5.2 Tumour necrosis factor-α

TNF-α is a pleotropic cytokine with a variety of effects in normal physiology and in immunity to a variety of infectious agents (Vassalli, 1992). Activated macrophages are a major source of TNF-α *in vivo*, however NK cells, T cells and mast cells also produce this factor (Beutler and Cerami, 1989). TNF-α occurs as a soluble or cell surface membrane-bound homotrimer and mediates its action through 2 distinct 55 kDa

(TNF-RI) and 75 kDa (TNF-RII) cell surface receptors (Tartaglia and Goeddel, 1992). The role of each receptor is unclear, however there is some evidence for involvement of both receptors in TNF-α induced cytotoxicity and LPS induced toxic shock (Pfeffer *et al.*, 1993; Bigda *et al.*, 1994). Studies by Wong *et al.*, (1992) indicate that antiviral activity of TNF-α is signalled through TNF-RI.

TNF-α exhibits direct anti-viral activity against a wide range of RNA and DNA viruses *in vitro* (Mestan *et al.*, 1986; Wong and Goeddel, 1986; Wong *et al.*, 1988). These antiviral effects of TNF-α include the inhibition of viral replication in infected cells, protection of uninfected cells from infection and killing of infected cells. These effects are enhanced synergistically by IFN-γ (Wong and Goeddel, 1986; Wong *et al.*, 1988). The mechanism by which TNF-α mediates its direct antiviral effect is unclear. TNF-α mediates cytotoxicity toward tumour cells by inducing these cells to undergo apoptosis (Dealtry *et al.*, 1987; Laster *et al.*, 1988) or generate toxic free oxygen radicals (Wong and Goeddel, 1988). These mechanisms may also mediate the antiviral effects of TNF-α. In addition, TNF-α can inhibit viral gene transcription *in vitro* thereby impairing virus growth (Feduchi *et al.*, 1989; Lucin *et al.*, 1994).

TNF-α has also been shown to exert antiviral effects *in vivo*. Administration of recombinant TNF-α limits the replication or pathology associated with Thieler's virus (Paya *et al.*, 1990), HSV-1 (Rossol-Voth *et al.*, 1991), Friend murine leukaemia virus (Johnson *et al.*, 1988) and encephalomyocarditis virus (Sriram *et al.*, 1991). In contrast, systemic administration of TNF-α or TNF-α neutralising antibodies had no effect on LCMV infection (Klavinskis *et al.*, 1989; Leist and Zinkernagel, 1990). *In vivo* TNF-α may mediate its antiviral effects directly by inhibiting virus replication in cells as described above. Alternatively, TNF-α may activate NK cells, macrophages or T cells to effector function (reviewed in Vassalli, 1992). Recombinant vaccinia virus encoding TNF-α was found to be markedly attenuated *in vivo* (Sambhi *et al.*, 1991). This effect apparently occurred independently of lymphocytes, as SCID mice recover from infection with this virus (J. Ruby, JCSMR, personal communication). This

finding suggests that the adaptive immune system may not be essential for the antiviral effect of TNF-α therapy .

1.5.3 Lymphotoxin (TNF-β)

Lymphotoxin (LT) is a cytokine which has a major role in lymphoid cell development and also exhibits antiviral activity. The expression of LT is largely restricted to activated lymphocytes (Paul and Ohara, 1988). Recently, mice lacking LT were found to lack secondary lymphoid tissues (Togni *et al.*, 1994), indicating a role for this factor in lymphocyte development. LT and TNF-α are related molecules exhibiting limited but significant sequence homology (Pennica *et al.*, 1984). Despite the lack of high sequence conservation, LT and TNF-α bind to the same receptors (Aggarwal *et al.*, 1985) and appear to mediate similar effects in target cells (Ruddle, 1992).

In light of these observations, it is not surprising that LT also exhibits antiviral activity against a number of RNA and DNA viruses (Aderka *et al.*, 1985; Wong and Goeddel, 1986). Furthermore, the antiviral activity of LT is augmented synergistically by IFN-γ (Wong and Goeddel, 1986). LT can also induce apoptosis (Schmid and Rouse, 1992) which may account for its direct antiviral activity. The *in vivo* relevance of LT in antiviral immune responses is unclear, however its role in lymphoid cell development suggests that LT may be involved in the activation of T cells as well as acting as an effector molecule.

1.5.4 Nitric oxide

Nitric oxide (NO) has many functions in mammals, including neural signalling, maintenance of vascular tone and host defence against a variety of infectious organisms (reviewed in Nathan, 1992). NO has been shown to limit tumour cell replication (Drapier and Hibbs Jr, 1986; Kwon *et al.*, 1991) and is toxic to a number of parasites (Adams *et al.*, 1990; Green *et al.*, 1990), bacteria (Stuehr and Marletta, 1987) and helminths (James, 1989). Recently, NO has been shown to inhibit replication of a number of DNA viruses such as ectromelia, vaccinia (Karupiah *et al.*, 1993b; Harris *et al.*, 1995; Karupiah and Harris, 1995) and HSV-1 (Croen, 1993). NO mediates

these effects by inactivating enzymes with iron-sulphur centres by forming nitrosyl-iron sulphur complexes (Lancaster and Hibbs, 1990; Pellat *et al.*, 1990). This process inhibits key enzymes in the Krebs cycle (*cis*-aconitase (Drapier and Hibbs Jr, 1986) and mitochondrial electron transport pathway (NADH: ubiquinone oxidoreductase and NADH: succinate oxidoreductase; (Granger *et al.*, 1980; Granger and Lehninger, 1982; Stuehr and Nathan, 1989). In addition, NO inhibits the activity of ribonucleotide reductase, a rate limiting enzyme in the synthesis of deoxynucleotides. Inactivation of this enzyme by NO prevents the replication of pathogens and tumour cells (Kwon *et al.*, 1991; Lepoivre *et al.*, 1991). NO may also mediate toxic effects by reaction with superoxide anion which generates the highly reactive and toxic hydroxyl radical (Ischiropoulos *et al.*, 1992).

NO is generated by the enzyme nitric oxide synthase (NOS). The enzyme catalyses the oxidation of L-arginine to L-citrulline and NO and is inhibited by L-arginine analogues such as N^G-monomethyl-L-arginine (L-NMA) and amino guanidine (Hibbs *et al.*, 1987; Marletta *et al.*, 1988). Three isoforms of NOS have been described and these are categorised into two groups based on their mechanism of activation (Nathan, 1992). Macrophage derived NOS (iNOS) is inducible by LPS or IFN-γ (Stuehr and Marletta, 1987) and is active independent of elevated intracellular calcium ion concentration (Cho *et al.*, 1992). Other cytokines, such as TNF-α, IFN-α, IFN-β and IL-1 alone do not stimulate macrophages to express iNOS, however these factors have been shown to synergise with IFN-γ or LPS to enhance NO production (Ding *et al.*, 1988). Human monocytes or monocyte-derived macrophages also appear to produce NO in response to alternative stimuli including some tumour cells (Isobe and Nakashima, 1993; Zembala *et al.*, 1994) and viral peptides (Mollace *et al.*, 1993; Pietraforte *et al.*, 1994). In contrast, brain and endothelial cells express different forms of calcium dependent NOS which are expressed constitutively (cNOS). In general, cNOS activity is not augmented by agents such as LPS or cytokines, but rather by agents such as excitatory amino acids, bradykinin, leukotrienes, thrombin and calcium ionophore (Braciale *et al.*, 1987; Moncada *et al.*, 1991).

1.5.5 Antibody

Antibodies exhibit antiviral activity by binding and neutralising infectious virus. Virus neutralisation by antibody is a complex phenomenon. It occurs via a number of mechanisms which may prevent virus attachment to, or entry of virus into, target cells, or subsequent events in virus replication (reviewed in Dimmock, 1993). In addition, antibody facilitates the specific recognition of virus infected cells by cytotoxic cells in a process described as antibody dependent cellular cytotoxicity (ADCC; Hashimoto *et al.*, 1983; Alsheikhly *et al.*, 1984; Ljunggren *et al.*, 1987)). Cells expressing receptors for the Fc region of antibodies, such as activated macrophages, NK cells and some polymorphonuclear cells, are capable of mediating ADCC (Hashimoto *et al.*, 1983). Interaction of these cytotoxic cells with virus infected cells via antibody - antigen ligation induces the lysis of target cells.

The relative importance of antibody in resistance to primary viral infection is variable. Resistance to primary infection with yellow fever, polio or coxsackie viruses requires circulating antibody, possibly to reduce viraemia (Mims, 1987). In contrast, the importance of antibody during primary infections with vaccinia or HSV is limited (Mims, 1987; Novembre *et al.*, 1989; Schmid and Rouse, 1992). In marked contrast, antibodies play an important role in the immune response to secondary or subsequent infections (Kris *et al.*, 1985). The presence of antibody at the time of infection facilitates a very rapid response which occurs well before other immune effector functions (such as macrophages, CTL) are activated.

1.5.6 Complement- Alternative and classical pathways

The complement system is a multistep, multiprotein effector mechanism which, upon activation, leads to a breakdown in the integrity of cell surface membranes and the death of affected cells or to the inactivation of enveloped viruses (Liszewski and Atkinson, 1993). Two modes of complement activation have been described. In the classical pathway, complement is activated by IgM or IgG antibody bound to the surface of microorganisms or infected host cells. The antibody binds the first complement protein which leads to a cascade of proteolysis affecting the

remaining complement proteins. The cascade culminates in the formation of the membrane attack complex (MAC) from several complement proteins which leads to lysis of affected cells. The complement cascade can also be activated at an intermediate step (the alternative pathway) which also leads to the formation of the MAC. In contrast to the classical pathway, the alternative pathway is activated in the absence of antibody. Rather, the deposition of the complement 3b (C3b) protein in an 'activating surface' such as the surface of an infecting bacteria or virus leads to the activation of the complement cascade and the formation of the MAC. A number of complement proteins have other roles in the immune system. Proteins such as C3b facilitate opsonisation of microorganisms by phagocytes while others including C3a and C5a act as leucocyte chemoatractants and promote inflammation (Frank and Fries, 1991).

Complement has been shown to be involved in the immune response toward a number of viruses. Mice depleted of complement by treatment with cobra venom factor suffer more severe infections with Sindbis or influenza viruses (Hicks *et al.*, 1978; Hirsh, 1982). Complement may exert its antiviral effect by lysing infected cells. For example cells infected with Sindbis, VSV, measles or respiratory syncytia viruses activate complement via the alternative pathway which leads to their lysis (Hirsh, 1982). The binding of the C3b protein to viruses can also augment their uptake and destruction by macrophages (Frank and Fries, 1991). A number of viruses such as vaccinia and herpes simplex virus are able to evade the effects of complement by encoding proteins which inhibit the activation of the complement cascade (reviewed in Fishelson, 1994; Issacs and Moss, 1995). This finding suggests that evasion of the antiviral effects of complement may be important to the persistence of these viruses.

Complement may also be involved in the pathogenesis of viral infections. For example, viruses such as HIV can utilise bound C3b to augment its uptake by macrophages and facilitate infection of these cells (reviewed in Marschang *et al.*, 1994). Viruses may also use cell bound complement proteins as cellular receptors which allow infection of these cells. This mechanism is employed by Epstein Barr virus which uses C2 protein

present on B cells to bind to and subsequently infect these cells (Fishelson, 1994).

1.6 THE RETROVIRUSES

1.6.1 Definitions and taxonomy

The retroviruses are small (60-120 nm) enveloped RNA viruses with a unique defining replicative cycle in which the RNA genome is transcribed into DNA and then integrated into the genome of the infected cell (Teich *et al.*, 1982; Francki *et al.*, 1991; Garfinkel, 1992; Poulet *et al.*, 1994). Retroviruses are a ubiquitous family of viruses which have been isolated from a multitude of vertebrate animals including mammals, amphibians and fish as well as invertebrates such as molluscs and even fungi (Teich *et al.*, 1982; Garfinkel, 1992; Poulet *et al.*, 1994). Many of these viruses are exogenous infectious agents, however a number exist as endogenous proviruses in the germ line and are inherited as Mendelian genes (Teich *et al.*, 1982).

Up until 1991, the family *Retroviridae* was divided into three subfamilies, *Oncornavirinae, Lentiviranae* and *Spumaviranae,* based on virion structure and pathogenicity (Murphy and Kingsbury, 1990). These classifications were not supported by analysis of retroviral genetic structure and nucleotide and amino acid sequence comparison, which led to a re-classification in 1991. The family *Retroviridae* was divided into seven genera which are *Lentivirus, Spumavirus* and five other as yet unnamed genera, which contain members of the former *Oncornavirinae* subfamily (Teich *et al.*, 1982; Francki *et al.*, 1991). Examples of retroviral genera and associated diseases are listed in Table 1.1.

1.7 RETROVIRAL DISEASES

1.7.1 Neoplasia

A common outcome of many retroviral infections is the generation of neoplasia. Retroviral induced malignancies commonly occur as leukaemias, lymphomas, sarcomas, and liver, kidney or mammary carcinomas (Teich *et al.*, 1982). Retroviruses can be divided into two classes based on the process of carcinogenesis. Non-acute transforming

Table 1.1 *Classification of the family* **Retroviridae**[a]

Genus[b]	Species[c]	Associated disease
Murine leukaemia virus-related group	Murine leukaemia virus: Rauscher, Friend, Moloney, Gross, LP-BM5	Neoplasia, immunodeficiency, neurological disorders, anaemia
Mammalian type B oncornavirus group	Mouse mammary tumour virus (MMTV)	Mammary carcinoma, T cell lymphoma
Type D retrovirus group	Mason-Pfizer monkey virus Squirrel monkey virus	Immunodeficiency
Avian type C group	Avian leukosis virus Rous sarcoma virus	Neoplasia, osteopetrosis, immunological disorders, Immunodeficiency
Human T cell lymphotropic virus (HTLV) - Bovine leukaemia virus (BLV) group	HTLV-I, II Simian T cell lymphotropic virus BLV	T and B cell lymphomas, neurological disorders, immunodeficiency
Lentivirus	Human immunodeficiency virus-I, II (HIV-I,-II) Simian immunodeficiency virus (SIV) Caprine arthritis encephalitis virus (CAEV) Equine infectious anaemia virus (EIAV) Feline immunodeficiency virus (FIV)	Immunodeficiency, autoimmune degenerative, neurological and other diseases
Spumavirus	Human foamy virus Simian foamy virus Feline foamy virus	None known

Table 1.1 (cont)

a) Adapted from Francki *et al.*, (1991) and Coffin, (1994)

b) A number of genera do not yet have approved international names. These genera are given English vernacular names and are written in normal text. Genera with approved names are shown in italics

c) Examples of each genera are shown

retroviruses induce neoplasia via insertional inactivation of cellular genes or insertional activation of proto-oncogenes. Carcinogenesis by acute transforming viruses involves the transduction of cellular proto-oncogenes, the transactivation of cellular genes by virus encoded peptides or the production of mitogenic viral peptides (reviewed in Bishop and Varmus, 1982; Kung *et al.*, 1991; Tsichilis and Lazo, 1991; Fan, 1994).

Central to the replicative cycle of retroviruses is the integration of the viral genome into the host genome. The insertion of retroviral sequences may lead to inactivation of host genes. The inactivation of genes involved in inhibiting cellular proliferation may lead to neoplasia. Insertional mutation of the cellular anti-oncogene p53 has been observed in tumours produced in mice infected with Abelson (Wolf and Rotter, 1984) or Friend murine leukaemia viruses (Mowat *et al.*, 1985; Chow *et al.*, 1987). Malignancies can also arise following retroviral provirus integration adjacent to cellular proto-oncogenes. The activation of these genes by promoter or enhancer elements in the retroviral LTR region is a major cause of malignancy associated with a wide range of retroviruses (Kung *et al.*, 1991). Oncogenesis by non-acute transforming viruses via insertional inactivation or activation of cellular genes is a relatively long process and may take many months to years to occur. Furthermore, these viruses do not induce transformation *in vitro*. This may reflect the often random nature of proviral DNA integration (Shih *et al.*, 1988) and the requirement for other events such as chromosomal translocation (Nottenburg *et al.*, 1987; Lazo and Tsichlis, 1988) to contribute to oncogenesis.

On the other hand, acute transforming retroviruses induce transformation over a short period of time (days to weeks) by transduction of cellular oncogenes. This involves the capture of cellular genes by the retrovirus via recombination between the viral and host genomes (Tsichilis and Lazo, 1991). The transduction of host genes involved in the control of cellular proliferation (proto-oncogenes) often results in neoplasia, as the acquired genes are often refractory to host regulatory mechanisms. This may be due to mutations in the captured gene or the lack of host control over gene expression driven by the viral LTR promoter.

The spleen focus forming virus of Rauscher and Friend murine leukaemia virus complexes encode a mutated viral envelope protein which induces a marked proliferation of cells expressing the erythropoietin receptor, such as progenitor erythroid cells (Ruta and Kabat, 1980; Li *et al.*, 1990). The viral protein alone is incapable of inducing transformation, however after 3 to 4 weeks, monoclonal erythroid tumours emerge. Events such as insertional activation of *spi*-1 transcription factor (Moreau-Gachelin *et al.*, 1988) and inactivation or deletion of p53 (Mowat *et al.*, 1985; Chow *et al.*, 1987) are also required for leukaemogenesis.

Retroviruses may also induce carcinogenesis via transactivation of cellular genes by viral gene products. The tax protein produced by the human T lymphotropic virus I upregulates a number of genes involved in the proliferation of T cells. These genes include IL-2, IL-2 receptor, GM-CSF, and *c-fos* (reviewed in Green and Chen, 1994). Although the process of immortalisation by HTLV-I is not completely understood, tax is thought to play a role via the chronic stimulation of these genes (Yodoi and Uchiyama, 1992).

1.7.2 Non-neoplastic retroviral diseases

Many retroviral infections lead to disease without inducing transformation. These can be broadly categorised as immunosuppressive, neurological, anaemia and wasting diseases (Table 1.1). Perhaps the most widely studied of these disorders is immunosuppression. Infection with lentiviruses, MuLV, HTLV or Mason-Pfizer viruses induce various forms of impaired immune responses (reviewed in Dent, 1972; Bendinelli *et al.*, 1985; Specter and Friedman, 1985). Retroviral infections have been shown to impair a range of immune effector functions, such as lymphocyte proliferation, antibody production, cytokine production and CTL activity. As a result, infected animals exhibit impaired resistance to other infectious agents (Bendinelli *et al.*, 1985; Levy, 1993). Retroviruses may induce immunosuppression in a number of ways. Envelope glycoproteins of MuLV, HTLV and HIV have all been shown to impair T cell function *in vitro* by inhibiting signal transduction by cell surface receptors, which may suppress proliferative or other signals (Ruegg *et al.*, 1989b; Ruegg *et al.*,

1989a; Kadota *et al.*, 1991). Furthermore, some retroviral proteins, such as HTLV encoded tax or HIV encoded tat, may alter cellular responses to a range of stimuli (Rosenblatt *et al.*, 1995). Alternatively, loss of immune cells, such as $CD4^+$ T cells, as found in HIV infection, may lead to suppressed immune responses (Zinkernagel and Hengartner, 1994).

A common occurrence in retrovirus infected animals is a wasting syndrome characterised by gradual, prolonged loss of weight and condition. Examples can be found in many retroviral genera, however this phenomenon is particularly common amongst animals infected with lentiviruses including HIV, SIV, EIAV, maedi-visna virus and CAEV (Narayan and Clements, 1989). The cause is largely unknown, however the symptoms are similar to the weight loss induced by chronic treatment with TNF (Vassalli, 1992). As TNF is overexpressed in some retroviral diseases such as HIV (Lahdevirta *et al.*, 1988), it has been suggested that wasting associated with retroviral infection is due to TNF mediated effects (Matsuyama *et al.*, 1991).

Neurological disorders are also associated with retroviral infections. These include HIV-associated neuromuscular disease, encephalopathy and dementia (Wiley and Nelson, 1990), HTLV-I-induced tropical spastic paraparesis (Roman and Osame, 1988) and paralysis of mice infected with Cas-Br-E MuLV (Gardner *et al.*, 1973). The mechanisms of neuropathogenesis in retroviral infections have not been clearly defined. These diseases may be due to inflammation of the central nervous system (CNS), neurotoxic effects of retroviral proteins or cytokines produced by cells within the CNS (Wiley and Nelson, 1990; Levy, 1993).

Retroviruses such as EIAV, FeLV, ALV, R-MuLV and F-MuLV also induce anaemia, which can take several forms depending on the virus. Anaemia associated with FeLV infection is characterised by aplasia due to the destruction of erythroid stem cells (Hardy, 1993). Alternatively, anaemia may be hypoplastic, due to inefficient maturation of haematopoietic stem cells, as found during ALV, R-MuLV, F-MuLV infections (Teich *et al.*, 1982), or haemolytic, due to destruction of infected precursor or mature red blood cells by antiviral immune responses, as seen following EIAV infection of horses (Sentsui and Kono, 1987).

It is worth noting that not all retroviruses are associated with disease. Viruses in the genus *Spumavirus,* as well as some endogenous MuLV, are non-pathogenic (Kozak and Ruscetti, 1992; Loh, 1993). Endogenous MuLV replicate at very low levels and persist via vertical transmission (Kozak and Ruscetti, 1992). These viruses may be the perfect parasites, as they persist through generations with minimal cost to the host or their own propagation.

1.8 THE MURINE LEUKAEMIA VIRUSES

Murine leukemia viruses (MuLV) are a major species within the mammalian type C retrovirus group. Most MuLV occur as non-productive endogenous viruses incorporated in the mouse genome. Some inbred strains of mice, such as AKR and C58, spontaneously produce these viruses from very early in life (Pincus, 1980). In most other strains of mice, activation of endogenous viruses occurs at low incidence, however mutagenic agents such as X-rays or carcinogens can significantly increase the rate of activation (Ribacchi and Giraldo, 1966; Zilber and Postnikova, 1966).

MuLVs can be divided into different classes based on their ability to infect cells of different species (reviewed in Hunter and Swanstrom, 1990). Ecotropic MuLV efficiently infect mouse cells but poorly infect cells from other animal species (Levy, 1974). Xenotropic MuLV are able to infect and replicate in cells of non-murine animals but do not efficiently infect mouse cells (Levy, 1973; reviewed in Levy, 1978). Replication competent MuLV, which differ from ecotropic and xenotropic viruses in host range, are termed polytropic viruses. A number of these viruses form foci on mink cell monolayers and have therefore been called mink cell focus (MCF) forming viruses (Fischinger *et al.*, 1975; Hartley *et al.*, 1977). Amphotropic viruses have been isolated from wild mice but are not found in laboratory mice. These viruses replicate in both murine and non-murine cells but differ from polytropic viruses in host range (Hartley and Rowe, 1976). The host range of MuLV is predominantly determined by the ability of major envelope glycoprotein (gp70) to bind to different cellular receptors (Kozak, 1983). The ecotropic MuLV receptor has recently been identified as an amino acid transporter (Kim *et al.*, 1991).

A number of highly pathogenic MuLV have been isolated over the last 50 years. These viruses were isolated by passage of leukaemic cells or cell-free leukaemic material in mice. Examples include Gross, Kirsten, Duplan-Laterjet (LP-BM5), Friend, Rauscher and Moloney MuLV viruses. Many of these have been found to be mixtures of ecotropic, polytropic and xenotropic viruses (reviewed in Kozak and Ruscetti, 1992). These viruses cause a wide range of diseases in mice, including T and B cell lymphomas, lymphoid, myeloid and erythroid leukaemias, neurological disorders, sarcomas and immunosuppression (Teich *et al.*, 1982; Kozak and Ruscetti, 1992). The relevance of these viruses in nature appears to be limited as they are difficult to transmit horizontally and may therefore may have disappeared with the death of the original animal.

1.9 RAUSCHER MURINE LEUKAEMIA VIRUS

1.9.1 R-MuLV and F-MuLV

The experiments described in this thesis examined the effect of R-MuLV infection on the immune system of inbred mice. It is therefore important to review the mechanisms of resistance and susceptibility to R-MuLV infection, with particular emphasis on the role of the immune response to R-MuLV. While the pathogenesis of R-MuLV infection and disease has been investigated for over 30 years, the body of information gathered about this virus is dwarfed by studies of the closely related F-MuLV. Genetic, morphological and immunologic studies show that the anaemia strain of F-MuLV (F-MuLV-A) is closely related to R-MuLV. These viruses produce very similar disease patterns in mice (Teich *et al.*, 1982). The polycythaemia strain of F-MuLV (F-MuLV-P) is also related to R-MuLV, however more distantly than F-MuLV-A (Wolf *et al.*, 1983; Bestwick *et al.*, 1984; Wolf *et al.*, 1985; Kabat, 1989). Furthermore, the disease produced by F-MuLV-P is slightly different to R-MuLV, as it involves a hypervolaemic polycythaemia, as opposed to anaemia (Teich *et al.*, 1982). Where studies of R-MuLV infection are lacking or inconclusive, studies using F-MuLV-A or the related F-MuLV-P will be described.

1.9.2 Pathogenesis of R-MuLV

Rauscher MuLV is a murine type C oncorna virus discovered by (and named after) F.J. Rauscher in the early 1960's (Rauscher, 1962). Infection of susceptible mice, such as BALB/c, A/J, SJL/J or NIH inbred Swiss, results in a rapid, persistent viraemia, proliferation of progenitor erythroid cells, anaemia and splenomegaly (Rauscher, 1962; Boiron *et al.*, 1965). Depending on the infecting dose, mice die from 4-6 weeks p.i., usually due to rupture of the grossly enlarged spleen. Surviving mice begin to develop B and T cell lymphomas 3-6 months p.i. (Rauscher, 1962; Reddy *et al.*, 1980; de Both *et al.*, 1983) In marked contrast, resistant C57BL/6 mice exhibit a transient viraemia, low level proliferation of progenitor erythroid cells and slight splenomegaly (Rauscher, 1962; Pluznik and Sachs, 1964; Boiron *et al.*, 1965; Borella, 1969). Although these mice efficiently control R-MuLV infection, the appearance of lymphomas in infected mice indicate that the virus is not totally cleared (Boiron *et al.*, 1965; McCoy *et al.*, 1967).

The major targets of R-MuLV are the progenitor erythroid cells (Rauscher, 1962; Pluznik and Sachs, 1964), Infection of these cells augments the mitogenic signal delivered through the erythropoietin receptor which results in a marked increase in the erythroid stem cells ie. burst forming (BFU-e) and colony forming (CFU-e) units found in the bone marrow and spleen 7-14 days post infection (Okunewick and Phillips, 1973; Gallacchio and Murphy Jr, 1983). By three weeks post infection approximately 60% of the spleen of susceptible mice is comprised of progenitor erythroid cells (de Both *et al.*, 1978). These progenitor erythroid cells are dependent upon erythropoietin for replication *in vitro* (Gallacchio and Murphy Jr, 1983) and *in vivo* (Okunewick and Erhard, 1974), however they do not efficiently mature into red blood cells which results in the anaemia associated with R-MuLV infection (Brodsky *et al.*, 1967). In the first 3-4 weeks after infection these cells also have limited renewal capacity (Hess *et al.*, 1984), however by 4 weeks post infection immortal oligoclonal leukaemias arise.

Many other cells are targets of R-MuLV infection. Megakaryocytes are readily infected by R-MuLV resulting in thrombocytopoiesis through as yet undefined mechanisms (Grau *et al.*, 1986). The role of lymphoid cells in

the pathogenesis of R-MuLV infection is unclear. In addition to being major contributors to anti-viral immune responses, lymphocytes may also act as targets of virus replication. The level of R-MuLV infection of B cells has not been formally demonstrated, however it interesting to note that F-MuLV replicates in spleen B cells and, to a lesser extent, in spleen T cells. Furthermore, F-MuLV replication is enhanced by stimulation of B cell proliferation (Isaak *et al.*, 1979). B cells appear to be essential for F-MuLV disease as BALB/c mice depleted of B cells from birth are refractory to F-MuLV induced disease (Manning *et al.*, 1974). Indirect evidence for R-MuLV infection of B cells is shown by the presence of virus particles near B cells very early in R-MuLV infection (from 24 h p.i. Hanna *et al.*, 1970a). Also, pre-B cell lymphomas occur following R-MuLV infection (Reddy *et al.*, 1980). Interestingly, germ-free mice were also found to be more resistant to R-MuLV than normal mice or germ-free mice challenged with antigen prior to infection. This suggests that lymphocytes (or lymphoid precursors) are targets of virus replication (Hanna *et al.*, 1970b; Kouttab and Jutila, 1972). Early investigation of the role of T cells in R-MuLV infection was unclear as depletion of T cells from susceptible mice using polyclonal thymocyte antisera has been shown both to ameliorate (Siegel and Morton, 1970) or exacerbate (Hirsch and Murphy, 1968) R-MuLV induced disease. Recently, Ruprecht and Bronson, 1994) showed that athymic nude BALB/c mice (which lack T cells) exhibit a disease similar to euthymic mice. This observation demonstrates that T cells are not essential for the pathogenesis of R-MuLV infection in BALB/c mice.

1.9.3 R-MuLV genetics

R-MuLV is a complex of at least 4 different viruses. Three of these viruses are replication competent, while the remaining virus is replication defective. Two of the replication competent viruses are ecotropic viruses (R-MuLV-eco) that differ in their ability to induce syncytia in the XC plaque assay (Vogt, 1982). These two viruses are thought to cause lymphatic leukaemia late in R-MuLV infection. The other replication competent virus is a polytropic mink cell focus forming virus (R-MCF; (van Griensven and Vogt, 1980). This virus has been shown to cause

erythroleukaemia with a latent period of 3-6 months. The remaining virus is a replication defective spleen focus forming virus (R-SFFV), which rapidly causes the generation of foci of proliferating cells when injected into mice (Hess *et al.*, 1984; Bestwick *et al.*, 1985). Rauscher-SFFV encodes a mutant envelope glycoprotein, gp52 (Ruta and Kabat, 1980), which is thought to bind to the erythropoietin receptor of haematopoietic progenitor cells, stimulating them to proliferate (Li *et al.*, 1990). Whilst R-SFFV can induce splenomegaly and erythroproliferation when injected by itself, the R-MuLV-eco virus is also required for the transformation of progenitor erythroid cells (Hess *et al.*, 1984; Bestwick *et al.*, 1985).

Retroviral insertional activation of cellular oncogenes occurs during R-MuLV infection. The R-SFFV and F-SFFV genomes integrate adjacent to the *spi*-1 gene in a very high proportion of R-MuLV-induced tumours (Moreau-Gachelin *et al.*, 1990). Furthermore, transcription of the *spi*-1 gene is markedly upregulated in these tumours (Moreau-Gachelin *et al.*, 1990). The precise function of *spi*-1 protein (independently isolated and referred to as PU.1; Klemsz *et al.*, 1990) is unclear, however it has transcriptional activator activity and exhibits marked homology to the *ets* nuclear transcription factor (Klemsz *et al.*, 1990). *In vitro* studies suggest that PU.1 is involved in controlling the differentiation of Friend erythroleukaemia cells (Schuetze *et al.*, 1992), which raise the possibility that PU.1 may be involved in oncogenesis during R-MuLV infection.

Recent studies indicate that the integration of F-MuLV-eco leads to activation of the *Fli-1* gene in a large number of erythroleukaemias induced by F-MuLV-eco (Ben-David *et al.*, 1991). The *Fli-1* gene is also related to *ets* transcription factor and encodes a transcriptional activator (Zhang *et al.*, 1993). Spi-1 and Fli-1 proteins are functionally distinct and bind different DNA sequences (Zhang *et al.*, 1993). The role of *Fli-1* in oncogenesis during R-MuLV infection remains to be demonstrated.

1.10 RESISTANCE AND SUSCEPTIBILITY TO R-MuLV DISEASE

Infection of mice with R-MuLV begins a complex interplay between the virus genome, host genetic factors and the host immune response. A number of autosomal mouse genes have been shown to control R-MuLV

infection and disease *in vivo*. Tóth *et al.*, (1973) proposed that 2 genes, *Rv-1* and *Rv-2*, control various aspects of R-MuLV replication. Although these genes were incompletely characterised, it is thought that the genes described were actually the *Fv-1* and *Fv-2* genes, which had been identified earlier as controlling F-MuLV induced disease (Steeves and Lilly, 1977). *Rv* and *Fv* genes will be considered as identical in the following discussion.

1.10.1 Fv-1

The *Fv-1* gene affects the ability of a range of MuLV to integrate into the genome of infected cells (reviewed in Teich *et al.*, 1982). Cells expressing a resistant *Fv-1* phenotype are 10-100 times less sensitive to infection by some MuLV (Pincus *et al.*, 1971a; Pincus *et al.*, 1971b). The nature of *Fv-1* restriction is unclear, however several studies indicate that the interaction between *Fv-1* and the viral gag protein, p30, influence the integration of the viral genome into host genomic DNA (Gautsch *et al.*, 1978; DesGroseillers and Jolicoeur, 1983). There are several *Fv-1* alleles designated $Fv\text{-}1^{n}$, $Fv\text{-}1^{b}$ and $Fv\text{-}1^{nr}$. The $Fv\text{-}1^{n}$ and $Fv\text{-}1^{b}$ alleles are named after the prototypic strains of mice from whom they derive, NIH Swiss and BALB/c respectively (Pincus *et al.*, 1971a). N-tropic viruses replicate more efficiently in cells of the $Fv\text{-}1^{n/n}$ genotype while $Fv\text{-}1^{b/b}$ cells support the replication of B-tropic viruses. Strains of mice which are $Fv\text{-}1^{n/n}$ include NIH/N, DBA/2, C3H/He and AKR/N while strains such as A/J, C57BL/6 and B10.BR/J are $Fv\text{-}1^{b/b}$ (Pincus *et al.*, 1971a). A third *Fv-1* allele, $Fv\text{-}1^{nr}$, was found in 129, NZW and NZB mice and was found to convey at least a four fold increase in resistance to N-tropic MuLV (Rowe and Hartley, reported in (Steeves and Lilly, 1977). Most strains of R-MuLV are N-B tropic and replicate equally well in cells from mice which are $Fv\text{-}1^{n/n}$ or $Fv\text{-}1^{b/b}$ (Hartley and Rowe, 1975). Thus it seems unlikely that *Fv-1* genotype would affect R-MuLV infection and subsequent disease in inbred mice, although $Fv\text{-}1^{nr}$ 129 mice appear to be slightly more resistant than $Fv\text{-}1^{n}$ or $Fv\text{-}1^{b}$ mice.

1.10.2 Fv-2

The replication and mitogenic effects of SFFV of Rauscher and Friend MuLV are under the control of the *Fv-2* gene. The two alleles $Fv\text{-}2^r$ and $Fv\text{-}2^s$ confer resistance and susceptibility respectively (Odaka and Yamamoto, 1962; Lilly, 1970). Genetic studies have shown that the resistance mediated by *Fv-2* is dominant over susceptibility at the *Fv-1* locus and that susceptibility is dominant in mice with heterozygous *Fv-2* alleles (Lilly, 1970). The Fv-2 protein is thought to control the proportion of progenitor erythroid cells actively progressing through the cell replication cycle (Suzuki and Axelrad, 1980). As retroviruses require cells to be actively replicating to integrate into the host genome (Luciw and Leung, 1992), this may explain why different patterns of progenitor erythroid cell replication affect R-MuLV and F-MuLV replication and disease. The Fv-2 gene product influences the mitogenic signal propagated by the erythropoietin receptor following ligation by erythropoietin or binding of gp55 F-SFFV (Hoatlin *et al.*, 1990). Although similar studies have not been performed, it seems likely that R-SFFV activation of the erythropoietin receptor is also controlled by the Fv-2 protein.

Resistance to F-MuLV conferred by *Fv-2* was described by Stutman and Dupuy, (1972) as absolute, since treatment of $Fv\text{-}2^{r/r}$ mice (C57BL/10 and C57BL/6) with anti-lymphocyte antisera did not alter virus growth. In contrast, subsequent studies have shown that C57BL/6 mice depleted of T cells with Thy 1 specific mAb (van der Gaag and Axelrad, 1990) or athymic *nu/nu* C57BL/6 mice (Kitagawa *et al.*, 1986; Kitagawa *et al.*, 1993) exhibit marked sensitivity to F-MuLV infection and disease. These studies clearly demonstrate the requirement for T cells, and presumably T cell-dependent antiviral immune responses in the resistance to F-MuLV infection conferred by the $Fv\text{-}2^{r/r}$ genotype.

1.10.3 Genes affecting the immune response to R-MuLV

A number of other genes have been shown to control the pathogenesis of F-MuLV infection of mice including the *H-2*, *Rfv-3* and *Fv-3* genes. Although the function of these genes during R-MuLV infection is largely unexplored, the similarities between the disease induced by F-MuLV and

R-MuLV infection indicate that these genes may also play a role in resistance to R-MuLV infection and disease.

Genes within the H-2 locus influence leukaemogenesis associated with F-MuLV infection. Susceptible mice (*Fv-2*$^{r/s}$) with a *H-2*$^{b/b}$ genotype have a high incidence of recovery from F-MuLV induced disease 10-14 days post infection. In contrast, congenic mice with the *H-2*$^{d/b}$ or *H-2*$^{a/b}$ genotype rarely recover when infected with high doses of F-MuLV. These heterozygous mice do however, recover from challenge with low doses of virus while congenic homozygous *H-2*$^{a/a}$ and *H-2*$^{d/d}$ do not. These effects are controlled by 2 genes within the MHC named *Rfv-1* and *Rfv-2*. (Chesebro and Wehrly, 1978). *Rfv-1* has been mapped to the D region of the *H-2* complex (Chesebro *et al.*, 1974) and mediates its effect via the generation of antiviral CTL (Chesebro, 1990). The *Rfv-1* gene is responsible for resistance to high doses of virus in susceptible *H-2*$^{b/b}$ mice. The *Rfv-2* gene maps to the K and I region of the mouse *H-2* locus and confers resistance to low doses of F-MuLV in *H-2*$^{d/b}$ or *H-2*$^{a/b}$ mice. The mechanism of resistance mediated by *Rfv-2* has not been identified (Chesebro, 1990).

The *Rfv-3* gene also controls the ability of congenic susceptible mice to recover from F-MuLV induced disease (Chesebro and Wehrly, 1979). The presence of *Rfv-3*^{r} genotype correlates with a high incidence of recovery from F-MuLV induced disease by the F-MuLV susceptible genotype *Fv-2*$^{s/s}$, *H-2*$^{a/a}$ or *H-2*$^{b/b}$. The recovery mediated by the *Rfv-3* locus is associated with the production of neutralising anti-F-MuLV antibodies 10-14 days post infection (Chesebro and Wehrly, 1979; Doig and Chesebro, 1979). It has been suggested that these antibodies act to prevent cycles of re-infection in these mice, however this remains to be shown (Chesebro, 1990).

The *Fv-3* gene has been shown to regulate the immunosuppression associated with infection with F-MuLV virus (Kumar *et al.*, 1978a; Kumar *et al.*, 1978b). Resistance is not absolute as large doses of virus (>1500 focus forming units) appear to significantly reduce differences between *Fv-3*$^{s/s}$ and *Fv-3*$^{r/r}$ mice (Kumar *et al.*, 1978b). Studies by others have failed to show an effect of *Fv-3* (Chesebro and Wehrly, 1979; Morrison *et al.*, 1986;

Chesebro, 1990) which has led to some scepticism as to the independence of *Fv-3* from *Fv-2* (Chesebro, 1990). Other studies have indicated that immunosuppression associated with F-MuLV is regulated by genes within the *H-2* complex and that these genes map within *H-2D* subregion (Morrison *et al.*, 1986; Morrison *et al.*, 1987). Resistance or susceptibility to immunosuppression is conferred by the $H\text{-}2D^b$ or $H\text{-}2D^d$ genotype respectively.

1.11 THE IMMUNE RESPONSE TO R-MuLV

Infection of susceptible mice with R-MuLV is characterised by a weak antiviral immune response. Toth *et al.*, (1971a) showed that BALB/c mice generate only low levels of antiviral antibody following infection with R-MuLV. In addition, BALB/c mice challenged with low doses of live R-MuLV failed to generate a cellular immune response to R-MuLV antigens as measured by the macrophage migration inhibition test (Mortensen *et al.*, 1973). The lack of an effective immune response in R-MuLV infected BALB/c mice is not due to an inherent inability of susceptible mice to recognise R-MuLV antigens, as seen in some other virus infections (Zinkernagel and Doherty, 1979; Bennink, 1988) since BALB/c mice generate strong immune responses toward R-MuLV following treatment with various vaccine preparations (Fink and Rauscher, 1964; Mortensen *et al.*, 1973; Peters *et al.*, 1975; Kelloff *et al.*, 1976).

Passive transfer of spleen cells from R-MuLV-immune BALB/c mice showed that acquired immunity is dependent on $CD4^+$ and $CD8^+$ cells (Hom *et al.*, 1991). It appears that CMI may be important in acquired immunity to R-MuLV as immune mice have been shown to express strong anti-R-MuLV DTH (Peters *et al.*, 1975; Kelloff *et al.*, 1976) or are positive in the macrophage migration inhibition test toward R-MuLV antigens (Mortensen *et al.*, 1973). Moreover, passive serotherapy with hyperimmune anti-R-MuLV sera only partially protects against R-MuLV infection (Fink and Rauscher, 1964; Ruprecht *et al.*, 1990).

Resistant C57BL/6 mice generate a vigorous anti-R-MuLV immune response characterised by high, persistent levels of antibody (McCoy *et al.*,

1972; Ishimoto and Ito, 1973) and strong induction of cell mediated immunity (Mortensen *et al.*, 1973). The *in vitro* growth of R-MuLV in spleen cells from C57BL/6 and BALB/c mice are similar (Ishimoto *et al.*, 1971) which indicates that there is no significant inherent differences in virus productivity between susceptible and resistant mice. Infectious virus is rapidly lost from plasma and spleen of infected C57BL/6 mice (Pluznik and Sachs, 1964) which suggests that R-MuLV is cleared by anti-R-MuLV immune responses. Interestingly, the virus is not totally eliminated as cells expressing R-MuLV-eco have been detected in the spleen of infected mice at least 6 weeks post infection (Iwai *et al.*, 1994).

IFN-α and IFN-β inhibit R-MuLV replication *in vivo*. Significant reduction of splenomegaly and increased survival times have been demonstrated following multiple injection of these IFN-α/β or IFN-β however these treatments do not cure susceptible mice of R-MuLV (Gresser *et al.*, 1968; Tóth *et al.*, 1971a; Hekman and Trapman, 1985). Susceptible, BALB/c mice can be cured of R-MuLV infection by treatment with IFN-α coupled with low dose AZT therapy (Ruprecht *et al.*, 1990). This finding also suggests that treatment with IFN-α may be curative for mice given very low doses of virus as low dose AZT inhibits R-MuLV replication but does not lead to clearance of the virus (Ruprecht *et al.*, 1986). Several strains of mice that are resistant to R-MuLV produce high levels of IFN while susceptible mice produce only low levels (Glasgow and Friedman, 1969; Tóth *et al.*, 1971c). In addition, neutralisation of type I IFN with polyclonal rabbit anti-IFN α/β antibodies augment R-MuLV induced splenomegaly in susceptible mice (Inglot and Chudzio, 1977). Interestingly, high levels of IFN are produced by susceptible NZB mice following infection with R-MuLV which suggests that factors other than IFN are also involved in resistance to R-MuLV (Siegel *et al.*, 1973).

Type I IFN exert antiviral effects upon most MuLV. Early studies of the effects of IFN on AKR MuLV demonstrated that IFN prevented the production of infectious virus particles from chronically infected cell lines (Pitha *et al.*, 1976). Examination of the antiviral effects of type I IFN on R-MuLV replication indicated that IFN treatment acted at a post-transcriptional level (Billiau, 1978) and that the processing of some

viral peptides and subsequent assembly of infectious virions was inhibited in IFN treated cells (Shapiro *et al.*, 1977; Pitha *et al.*, 1979; Pitha *et al.*, 1980). In addition to direct antiviral effects on target cells, IFN may also stimulate a number of immune effector functions such as NK cell activity or MHC class I expression (see section 1.5.1). The relative importance of these effector mechanisms in R-MuLV infection remains to be elucidated.

A feature of R-MuLV infection is a profound suppression of immune responses to a variety of stimuli. The generation of antibody to a variety of antigens such as bovine serum albumin, sheep red blood cells and influenza virus haemagglutinin have been shown to be suppressed in R-MuLV infected susceptible mice (Siegel and Morton, 1966; Ceglowski and Friedman, 1968; Millian and Schaeffer, 1968). In addition, the proliferation of spleen T cells following stimulation with phytohaemagglutinin (PHA) or allogeneic spleen cells were suppressed in R-MuLV infected BALB/c mice (Häyry *et al.*, 1970). In contrast, the PHA stimulated proliferation of lymph node T cells is largely unaffected by R-MuLV infection (Häyry *et al.*, 1970). Several studies have shown that R-MuLV infection also inhibits the ability of dendritic cells to migrate and provide costimulation in mixed lymphocyte reactions (Gabrilovich *et al.*, 1993; Gabrilovich *et al.*, 1994). Resistant C57BL/6 mice (which are also *H-2D*^{b} and *Fv-3*$^{r/r}$) also exhibit suppressed antibody production following R-MuLV infection however the degree of suppression is small, transient and interestingly, correlates with the presence of virus in the plasma of infected mice (Borella, 1969; Seidel and Lauenstein, 1969).

The cause of R-MuLV induced immunosuppression is largely unknown. It has been suggested that the accumulation of progenitor erythroid cells in the spleen may suppress immune responses, however many responses are suppressed before large numbers of these cells accumulate in the spleen (Ceglowski and Friedman, 1968; Häyry *et al.*, 1970). Furthermore, leukaemogenesis does not appear to be necessary for the development of immunosuppression associated with F-MuLV infection (Lopez-Cepero *et al.*, 1988; Matteucci *et al.*, 1989). Fowler *et al.*, (1977) showed that disrupted R-MuLV was capable of suppressing PHA induced T cell proliferation *in vitro*. This observation suggests that virus infection is not required for

R-MuLV to suppress immune function. This finding is consistent with the report of Ruegg *et al.,* (1989a) who showed that the R-MuLV p15E protein can inhibit T cell proliferation *in vitro*. The importance of R-MuLV infection of immune cells in R-MuLV induced immunosuppression is not known.

1.12 SCOPE OF THIS THESIS

The replication of R-MuLV in inbred strains of mice is regulated by a number of autosomal genes. While some genes influence the ability of R-MuLV to replicate others modulate the antiviral immune response generated toward R-MuLV. The experiments in this thesis were designed to examine the role of the immune system in resistance or susceptibility to R-MuLV infection. For the purpose of comparison susceptible BALB/c and resistant C57BL/6 mice were used.

Immunosuppression is associated with many retroviral infections and is a potential mechanism of evading host immune responses. Experiments in Chapter 2 were designed to investigate the effect R-MuLV infection had on immune function. BALB/c mice were more susceptible to R-MuLV infection and exhibited greater immunosuppression than R-MuLV infected C57BL/6 mice. A number of possible mechanisms which led to reduced immune responsiveness were investigated.

Over the last 10 years it has become clear that cytokines play an integral role in the generation and effector phase of antiviral immunity. Experiments described in Chapter 3 examine cytokine expression during R-MuLV infection. The requirement of cytokines in resistance or susceptibility was determined using cytokine neutralising antibodies and mice lacking functional IFN-γ receptors. Since T cells are major producers of cytokines and influence the type of immune response generated the requirement of T cell subsets in resistance or susceptibility to R-MuLV was examined.

Nitric oxide has been shown to limit virus replication and inhibit the proliferation of rapidly dividing cells such as tumour cells and lymphocytes. The role of NO in R-MuLV infection was therefore examined. Experiments described in Chapter 4 showed that NO was produced during R-MuLV infection of both BALB/c and C57BL/6 mice. However, further experiments indicated that NO had little influence upon resistance or susceptibility to R-MuLV or upon immunosuppression associated with R-MuLV infection.

Chapter 2

R-MuLV induced immunosuppression

2.1 INTRODUCTION

With the advent of the HIV pandemic the immunosuppression associated with virus infections has become a subject of increasing research interest. While the retroviruses are the predominant family of viruses that cause immunosuppression (for review see Dent, 1972; Bendinelli *et al.*, 1985), many other viruses impair host immune responses. These include measles virus (Smithwick and Berkovich, 1969), lactate dehydrogenase virus (LDV; Howard *et al.*, 1969), LCMV (Mims and Wainwright, 1968) and human cytomegalovirus (Rinaldo *et al.*, 1980). A wide range of immune functions have been shown to be suppressed, including the production of antibody, generation of cytotoxic T cells, lymphocyte proliferation, the generation of delayed type hypersensitivity, allograft rejection and production of cytokines (reviewed in Specter and Friedman, 1985; McChesney and Oldstone, 1987).

There are many potential mechanisms by which the immune system can be suppressed during virus infections, however it is often difficult to pinpoint defects in the cascade of events which occur during an immune response. Nevertheless, putative mechanisms of immunosuppression can be classified into 2 broad categories. Firstly, infection of a class of immune cells can lead to an intrinsic defect in the function of infected cells. This defect may be due to modified responses to stimuli such as ligation of antigen receptor on lymphocytes (Mizuochi *et al.*, 1990). Alternatively, infected cells may be lost through a number of mechanisms including lysis, as in the case of lytic viruses such as LDV (Stueckemann *et al.*, 1982). Immune cells may also be deleted by effective immune responses targeted toward virus infected cells, which occurs during LCMV infection (Zinkernagel and Hengartner, 1994) or apoptosis of infected cells which has been observed during HIV infection (Groux *et al.*, 1992; Gougeon *et al.*, 1993). Secondly, immunosuppression may be caused in an indirect manner. In this scenario, uninfected cells are affected due to the altered function of virus infected immune cells. Alternatively, the production of immunosuppressive factors such as

nitric oxide, prostaglandins or cytokines by dysregulated infected cells, or as part of antiviral immune responses, may lead to immunosuppression.

The cellular events leading to suppressed immune function during retrovirus infection are largely unresolved. This is due, at least in part, to the replication of many retroviruses in immune cells which complicates the interpretation of observations of immune function during retrovirus infection. Retrovirus encoded proteins have been shown to alter a number of immune responses. The envelope components of murine leukemia viruses (p15E) and the human T lymphotropic virus (gp21E) have been shown to impair lymphocyte proliferation and chemotaxis of macrophages or monocytes (Snyderman and Ciancolo, 1984; Ruegg *et al.*, 1989a; Ruegg *et al.*, 1989b). Studies by Kadota *et al.*, (1991) indicate that these proteins downregulate protein kinase C signalling pathways which are required for signal transduction from the cell surface receptors. A number of studies have shown that addition of purified protein or UV-inactivated virus is capable of mediating these effects (Fowler *et al.*, 1977; Ruegg *et al.*, 1989a; Ruegg *et al.*, 1989b). These observations suggest that immunosuppressive peptides do not have to be produced by infected cell to affect immune responses.

Infection of mice with R-MuLV disrupts a number of immune functions (Ceglowski and Friedman, 1968; Dent, 1972; Bendinelli *et al.*, 1985). In order to examine the immunosuppression associated with R-MuLV infection in greater detail, lymphocyte proliferation by cells from R-MuLV infected mice was studied *in vitro* using strains of mice with differing susceptibility to infection. R-MuLV infection of highly susceptible BALB/c and relatively resistant C57BL/6 mice rapidly led to impaired *in vitro* T and B cell proliferative responses. Potential mechanisms of immunosuppression were investigated.

2.2 MATERIALS AND METHODS

2.2.1 Mice

Mice were obtained from the Animal Services Division at the Australian National University and were raised under specific pathogen-free conditions. BALB/c and C57BL/6J mice were used at 6-11 weeks of age.

2.2.2 Virus

Rauscher MuLV was obtained from Dr C. Birch (Fairfield Hospital, Victoria Australia). R-MuLV was passaged by i.p. injection of 10^4 pfu of R-MuLV into BALB/c mice. After 21 days the mice were sacrificed and a single cell suspension was prepared from pooled spleens (20% w/v in DMEM supplemented with 20% FCS). The cells were removed by centrifugation at 500g for 10 min at 4°C and the supernatant was aliquoted and stored at -70°C. The titre of the stock was determined by XC plaque assay (Section 2.2.5). Virus stocks were diluted with gelatin saline to contain 10^4 XC pfu/ml before use. Mice were routinely infected with 10^4 pfu of virus, administered by i.p. injection.

2.2.3 Culture Media

Cells were grown in Dulbecco's modified Eagle's media (DMEM) containing a number of supplements. H16 media is composed of DMEM (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated FCS (Flow Laboratories, North Ryde, NSW, Australia), 10 mM HEPES, 2 mM L-glutamine, penicillin (30 mg/l), streptomycin sulphate (50 mg/l), neomycin sulphate (50 mg/l) and 50 µM 2-mercaptoethanol (Sigma Chemical Company, St. Louis, MO, USA).

Mixed lymphocyte culture medium (MLCM) consisted of H16 supplemented with glucose (4 g/l), folic acid (6 mg/ml), L-asparagine (36 mg/l), L-arginine (116 mg/l), sodium bicarbonate (2 g/l) and 1 mM sodium pyruvate.

2.2.4 Cells

SC-1 cells, a cell line derived from feral mouse embryos (Hartley and Rowe, 1975) and XC cells, a rat fibroblast tumour cell line (Svoboda *et al.*, 1963) were maintained in H16. These cells were passaged every 2-3 days by diluting the cells 1:10 into a new culture flask.

Spleen and lymph node cells were cultured in MLCM.

2.2.5 Virus Titration

Titration of R-MuLV used the XC plaque assay (Rowe *et al.*, 1970). SC-1 cells were seeded into each well of a 6 well plate (10^5 cells/well) and cultured for 24 h. Virus samples were serially diluted in H16 containing 8 µg/ml polybrene (Sigma) and 2 ml of each dilution was overlayed onto SC-1 cells. The media was replaced 24 hours later and, after a further 2 days, the cells were UV irradiated (15W lamp at 25 cm for 20 sec) and overlayed with $3.5x10^5$ XC cells/well. The media was replaced 24 h later and, after a further 2-3 days, the monolayers were stained with 0.1% crystal violet in 20% ethanol for 5 min and air dried. Plaques were counted using a dissecting microscope.

Spleens removed from mice infected with R-MuLV were stored individually at -70°C. Cell free virus was assayed by homogenising frozen spleen in 1 ml ice cold gelatine saline containing 20 mM HEPES for 15 sec in a polytron homogeniser. The homogenate was diluted to 3 ml final volume with gelatin saline and assayed for infectious virus using the XC plaque assay described above.

2.2.6 Infectious Centre Assay

R-MuLV infected spleen cells were titrated using the XC infectious centre assay (Melief *et al.*, 1975). Spleens from infected mice were aseptically removed and a single cell suspension was prepared in H16 containing 5% FCS. The cells were washed with H16/5% FCS adjusted to $5x10^7$ cells/ml and incubated with 25 µg/ml mitomycin C (Sigma) at 37°C for 30 min. The cells were washed, adjusted to 10^7 cells/ml and serially diluted in

H16 containing 8 µg/ml polybrene. Each dilution was overlayed onto SC-1 cells which had been seeded 24 h earlier into 6 well plates (10^5 cells/well). The media was changed 24 h later and the assay developed as for the XC plaque assay above (Section 2.2.5).

2.2.7 R-MuLV specific ELISA

A capture ELISA was developed to detect R-MuLV antigen. The ELISA employed goat polyclonal anti-R-MuLV serum derived from goat immunised with intact R-MuLV (Lot # 75S000297; Quality Biotech, Camden, NJ, USA), polyclonal goat anti-R-MuLV antisera that had been biotinylated (described below) and alkaline phosphatase conjugated streptavidin (Amersham Australia, North Ryde, NSW, Australia).

Polyclonal goat anti-R-MuLV sera was biotinylated as described in Harlow and Lane, (1988). Briefly, goat serum was biotinylated as follows. Serum was diluted to 1 mg/ml total protein in bicarbonate buffer (4.2 g/l; pH 8.5) and for each mg of serum protein 120 µg of succinimidobiotin (Sigma) dissolved in dimethyl sulphoxide was added. Biotinylation was performed at room temperature for 2 h in the dark. Unreacted succinimidobiotin was removed by washing the mixture three times with cold PBS by centrifugation over a Centricon 10,000 column (Amicon, CT, USA) according to the instructions provided by the manufacturer.

The R-MuLV ELISA used round bottomed 96 well ELISA plates (Dyantech Laboratories, VA, USA). Each well was coated with 50 µl of polyclonal anti-R-MuLV sera that had been diluted 1:800 in bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$ pH 9.6) and allowed to bind to the plate overnight at 4°C. Unbound antibody was removed by washing with PBS/0.1% Tween-20 (Sigma). Non-specific protein binding sites were blocked with 100 µl of 3% BSA (Sigma) for 1 h at 37°C. Plates were washed again and appropriate dilutions of virus containing samples were added to each well in 50 µl. Virus was allowed to bind for 2 h at room temperature and, after washing, 50 µl of 1:500 diluted biotinylated polyclonal goat anti-R-MuLV was added and allowed to bind for 90 min at room temperature. Bound biotinylated antibody was detected using

50 µl alkaline phosphatase conjugated streptavidin diluted 1:1000. The ELISA was developed using disodium *p* -nitrophenol phosphate (Sigma) at 1 mg/ml in 0.5 M diethanolamine (Fluka Chemika-Biochemika, Buchs, Switzerland), 25 mM $MgCl_2$, pH 9.6. Optical density (OD) was measured after 30 min using a Thermomax plate reader (Molecular Devices, Menlo Park, CA, USA). The OD was read at 410 nm with a reference at 650 nm. Non-specific development of the ELISA was determined by running the ELISA using 1% BSA in PBS instead of R-MuLV antigen. The endpoint of the ELISA was defined as the well with an OD greater than the mean background + (3x standard deviation of background).

2.2.8 Plasma isolation

Blood was obtained from mice via tail vein bleeding, collected, placed into a microcentrifuge tube containing heparin sulphate (5 U in 10 µl per 500 µl blood) and stored on ice. Blood cells were collected by centrifugation at 12,000g for 5 min at 4°C. The plasma was removed, aliquoted and stored at -70°C until assay for viral antigen or infectious virus.

2.2.9 Preparation of spleen and lymph node cells

Single cell suspensions from spleens or pooled brachial and inguinal lymph nodes were obtained by gently pushing organs through stainless steel mesh with a glass rod into MLCM. Erythrocytes were removed from spleen cell suspensions by water lysis. Cells were washed twice with MLCM prior to use.

2.2.10 Proliferation assays

T cell proliferation was assayed by stimulating spleen or lymph node cells with anti-CD3 antibody, clone 145-2C11 (Leo *et al.*, 1987), which was bound to round bottomed 96 well plates (Linbro, ICN Biomedicals, Irvine, CA, USA). The plates were previously coated with 125 ng of anti-CD3 antibody/well in 40 µl of PBS for 90 min at 37°C, washed twice with cold PBS. Plates were used either immediately or after storage in 100 µl

PBS/well at 4°C. Cells were cultured at $2x10^5$/well in 200 µl of MLCM. Replicate cultures were incubated for 72 h at 37°C and then labelled with 1 µCi of [^{3}H]-TdR (ICN Biomedicals) for a further 18 h. The cultures were harvested using an automatic plate harvester and counted using a betaplate liquid scintillation counter (Pharmacia LKB, Uppsula, Sweden).

Spleen B cell proliferation was determined by stimulation with LPS (*E. coli* strain 0128:B12, water-phenol extracted; Sigma). Cells were cultured at $2x10^5$ cells/well in 200 µl of MLCM containing 10 µg/ml LPS at 37°C. After 72 h incubation the cultures were labelled with 1 µCi of [^{3}H]-TdR for a further 18 h and harvested as above.

In some experiments $5x10^4$ spleen cells from uninfected or infected mice were added to an equal number of spleen cells from uninfected mice. The cells were cultured in MLCM and stimulated with immobilised anti-CD3 antibodies for 72 h at 37°C. Proliferation was measured by TdR incorporation following addition of 1 µCi of [^{3}H]-TdR (ICN Biochemicals) and culture for a further 18 h and harvested as above.

2.2.11 Antibodies

The following antibodies were used to determine the proportions of splenocytes expressing specific cell surface antigens: FITC conjugated anti-B220, clone RA3-6B2 (Coffman, 1982) was kindly supplied by Dr Philip Hodgkin (Division of Cell Biology, JCSMR) and used at a 1:100 dilution in PBS; biotinylated anti-Thy1.2, clone 30-H12 (Ledbetter and Herzenberg, 1979) was purchased from Becton Dickinson (Mountain View, CA, USA) and was used at 1:25, Streptavidin fluorescein (Amersham) was used at 1:40.

Supernatants of the hybridoma J11d (Bruce *et al.*, 1981) were generously provided by Dr Janet Ruby (Division of Cell Biology, JCSMR). The supernatants were used to lyse spleen cells expressing heat stable antigen (CD 24) using methods described in Section 2.2.14.

2.2.12 Immunofluorescence staining

5-10 x 10^5 cells were collected by centrifugation at 200g for 5 min at 4°C. Supernatants were aspirated and the cells were resuspended in 50 µl of diluted antibody. After 20-30 min incubation on ice, unbound reagents were removed by adding 500 µl of ice cold PBS and collecting cells as above. The cells were incubated on ice for a further for 20-30 min in 50 µl of diluted antibody or streptavidin fluorescein. Unbound reagents were removed by washing the cells with 500 µl of ice cold PBS. Cells were resuspended in 500 µl of cold PBS and stored on ice until assayed by flow cytometry. If flow cytometry was not performed until the next day, the cells were collected, resuspended in 500 µl of fixing buffer (16.83 g/l $NaH_2PO_4.2H_2O$, 3.85 g/l NaOH, 0.95% formaldehyde, 20 g/l glucose and 0.03% NaN_3) and stored at 4°C overnight in the dark.

2.2.13 Flow cytometry

Fluorescent labelled cells were analysed using a Becton Dickinson FACScan. FITC was excited using 100 mW laser light at 488 nm with FITC fluorescence emissions detected through a 530 nm band pass filter. 10,000 events were collected for each sample. Data was analysed using the Lysis II or Cell Quest programs (Becton Dickinson).

Side and forward scatter were used as criteria for gating of live cells. The proportion of brightly stained live cells was calculated relative to control cells stained with fluorochrome reagents. Calculations are based on data from 10,000 events.

2.2.14 Enrichment of T cells

Splenocytes were enriched for Thy 1.2^+ cells using the following protocol. 1-2x10^8 spleen cells prepared as in Section 2.2.9 were adjusted to a volume of 5 ml in cold MLCM and carefully layered onto 10 ml cold isotonic 32% Percoll (Pharmacia). The gradients were centrifuged for 25 min at 2500g at 4°C. Low density cells were removed using a Pasteur pipette and pelleted cells were washed in MLCM. These higher density cells were resuspended in 7 ml MLCM and incubated at 37°C with 2 ml J11d culture

supernatant for 15 min. J11d$^+$ cells were lysed by the addition of 1 ml rabbit complement (Cedar Lane Laboratories, Ontario, Canada) followed by incubation for a further 45 min at 37°C with gentle shaking every 15 min. Viable cells were isolated by centrifugation of the cells over a ficoll/metrizoate density gradient ρ=1.803 g/l (Davidson and Parish, 1975) at room temperature, 1500g for 20 min. The cells at the interface were removed, washed and resuspended in MLCM.

The enriched cells were then used in proliferation assays as described in Section 2.2.10. The proportion of Thy 1.2$^+$ cells was assayed using flow cytometry as described in Sections 2.2.12 and 2.2.13.

2.2.15 Statistical analysis

The relationship between different groups was determined using unpaired Student's *t* test. All quoted probabilities of significance use this test unless otherwise stated. As Student's *t* test is invalid where the standard deviations of two different samples are significantly different, Welch's *t* test was used in these instances. The difference between two groups was considered significant different if the probability of relatedness was less than 5% ie. $p<0.05$.

2.3 RESULTS

2.3.1 Growth of R-MuLV in BALB/c and C57BL/6 mice

In this thesis, R-MuLV infection of susceptible (BALB/c) and resistant (C57BL/6) strains of mice was studied. Although the growth of R-MuLV and disease pathology in these two strains has been described previously (Rauscher, 1962; Rauscher and Allen, 1964; Boiron *et al.*, 1965; Ishimoto *et al.*, 1971; Iwai *et al.*, 1994) it was necessary to determine the growth of R-MuLV and the resulting disease, using the conditions and virus available at the JCSMR.

Groups of BALB/c and C57BL/6 mice were injected i.p. with 10^4 pfu R-MuLV and a variety of parameters of virus growth were measured at 1, 2 and 3 weeks p.i. These included spleen weight, numbers of virus producing spleen cells, infectious virus in plasma and viral antigen in plasma and cell-free spleen extracts.

Infection of BALB/c mice with R-MuLV led to a rapid and marked increase in spleen weight which reached 10 times that of uninfected mice by 3 weeks p.i. (Table 2.1). Large numbers of virus-producing spleen cells were detected at 1, 2 and 3 weeks p.i. In contrast, infection of C57BL/6 mice induced a slight splenomegaly with a 2-3 fold increase in spleen weight at 2 and 3 weeks p.i. (Table 2.1). The numbers of virus expressing cells in spleens of infected C57BL/6 mice peaked at 1 week p.i., decreased thereafter and were much lower at all time points compared to BALB/c mice .

Infectious virus was readily detected in the plasma and cell-free extracts from spleens of infected BALB/c mice (Table 2.2). High levels of virus were recovered at 1, 2 and 3 weeks p.i. Viral antigen in plasma from infected BALB/c mice rapidly increased over the time points at which samples were taken. In marked contrast, the levels of infectious virus in plasma or cell-free spleen extracts from infected C57BL/6 mice were below the limit of detection of the XC assay (100 pfu/ml plasma or 100 pfu/g spleen respectively) at any time p.i. (Table 2.2). Low levels of viral

Table 2.1 *Splenomegaly and virus expressing cells during R-MuLV infection*

Mouse Strain	Weeks Post Infection	Mean Spleen Weight (mg)[a]	Spleen Infectious Centres[b]
BALB/c	Uninfected	120 ± 6	ND
	1	200 ± 8[c]	5.72 ± 0.06
	2	615 ± 63[d]	5.65 ± 0.10
	3	1285 ± 73[d]	5.60 ± 0.06
C57BL/6	Uninfected	94 ± 6	ND
	1	144 ± 35	3.88 ± 0.09
	2	219 ± 20[e]	1.49 ± 0.16[f]
	3	208 ± 26[e]	1.97 ± 0.60[g]

Groups of 4 BALB/c or C57BL/6 mice were given 10^4 pfu R-MuLV i.p. 1, 2 or 3 weeks previously. The spleen was taken from each mouse and weighed in tared sterile tubes containing 5 ml of H16 5% FCS. Single cell suspensions were prepared from each spleen and the numbers of virus expressing cells were assayed using the XC infectious centre assay described in Section 2.2.6.

a) Mean spleen weight of 4 mice ± SEM

b) Log_{10} infectious centres producing ecotropic R-MuLV/10^7 nucleated spleen cells. Data shown is mean ± SEM of 4 mice

c) $p<0.001$, versus spleen weight of uninfected BALB/c mice

d) $p<0.005$, versus spleen weight of uninfected BALB/c mice (Welch's *t* test)

e) $p<0.02$, versus spleen weight of uninfected C57BL/6 mice (Welch's *t* test)

f) $p<0.02$, versus number of virus producing spleen cells from C57BL/6 mice at 1 week p.i. (Welch's *t* test)

g) $p=0.05$, versus number of virus producing spleen cells from C57BL/6 mice at 1 week p.i. (Welch's *t* test)

Table 2.2 *Infectious virus and virus antigen recovered from R-MuLV infected mice*

Mouse Strain	Weeks Post Infection	Plasma R-MuLV[a]	Plasma antigen[b]	Spleen R-MuLV[c]
BALB/c	Uninfected	ND	40	ND
	1	4.51 ± 0.16	160 ± 44	6.39 ± 0.14
	2	4.70 ± 0.18	704 ± 157	6.28 ± 0.14
	3	4.67 ± 0.16	2048 ± 313	6.10 ± 0.18
C57BL/6	Uninfected	ND	40	ND
	1	<2	120 ± 55	<2
	2	<2	80 ± 10	<2
	3	<2	40	<2

Groups of 5 BALB/c or C57BL/6 mice were given 10^4 pfu R-MuLV i.p. on the appropriate week prior to virus assay. Each mouse was anaesthetised with 400 µl of avertin i.p. Blood was taken by cardiac puncture and placed in heparinised tubes. Plasma was separated from blood cells by centrifugation and assayed for viral antigen by ELISA or infectious virus by XC assay. A cell-free extract of each spleen was prepared and assayed for ecotropic virus using the XC assay described in Section 2.2.5.

a) Log_{10} pfu of ecotropic R-MuLV per ml of plasma. Data represent mean ± SEM of 5 mice

b) Endpoint of R-MuLV ELISA of R-MuLV antigen in plasma. Data represent mean ± SEM of 5 mice

c) Log_{10} pfu of ecotropic R-MuLV per gram of spleen. Data represent mean ± SEM of 5 mice

antigen were detected in plasma at 1 and 2 weeks p.i. which suggests that virus replication occurred at these times.

2.3.2 Proliferation of immune cells from R-MuLV infected mice

It has been known for some time that *in vitro* proliferative responses of immune cells from mice infected with R-MuLV are suppressed (Häyry *et al.*, 1970), however the mechanism of this immunosuppression has not been fully elucidated. In order to examine this further, an *in vitro* model was established to examine the proliferation of T or B cells from R-MuLV infected mice. Spleen and lymph node cells were prepared from mice infected with R-MuLV and their ability to proliferate in response to stimulation with anti-CD3 (T cells) or LPS (B cells) was determined.

Infection with R-MuLV had a marked effect of the proliferation of spleen T and B cells from both BALB/c and C57BL/6 mice. The proliferation of BALB/c spleen T and B cells was significantly depressed at 1 week p.i. and almost completely suppressed after infection for 3 weeks (Figure 2.1a, b). Spleen cells from infected C57BL/6 mice also exhibited decreased proliferative responses at 1 week p.i. At later time points, C57BL/6 spleen T cells proliferated to a greater extent than BALB/c T cells. At 2 weeks p.i. C57BL/6 T cell proliferation was approximately 35% of normal responses, while at 3 weeks p.i. proliferation was approximately 50% that of uninfected cells (Figure 2.1b). C57BL/6 spleen B cell proliferation was also suppressed by 1 week p.i. and remained so up to week 3 p.i. (Figure 2.1b).

In marked contrast to spleen cells, lymph node cells from R-MuLV infected mice exhibited unaltered proliferative responses to immobilised anti-CD3 antibodies (Figure 2.1c). Cells from both strains of mice proliferated to levels comparable with uninfected controls.

2.3.3 Proportion of T and B cells in cultured spleen cells

A feature of R-MuLV infection in BALB/c mice is a rapid accumulation of progenitor erythroid cells in the spleen (de Both *et al.*, 1978). Suppressed proliferative responses of spleen lymphocytes may thus reflect a decrease in the proportion of responsive cells rather than a defect

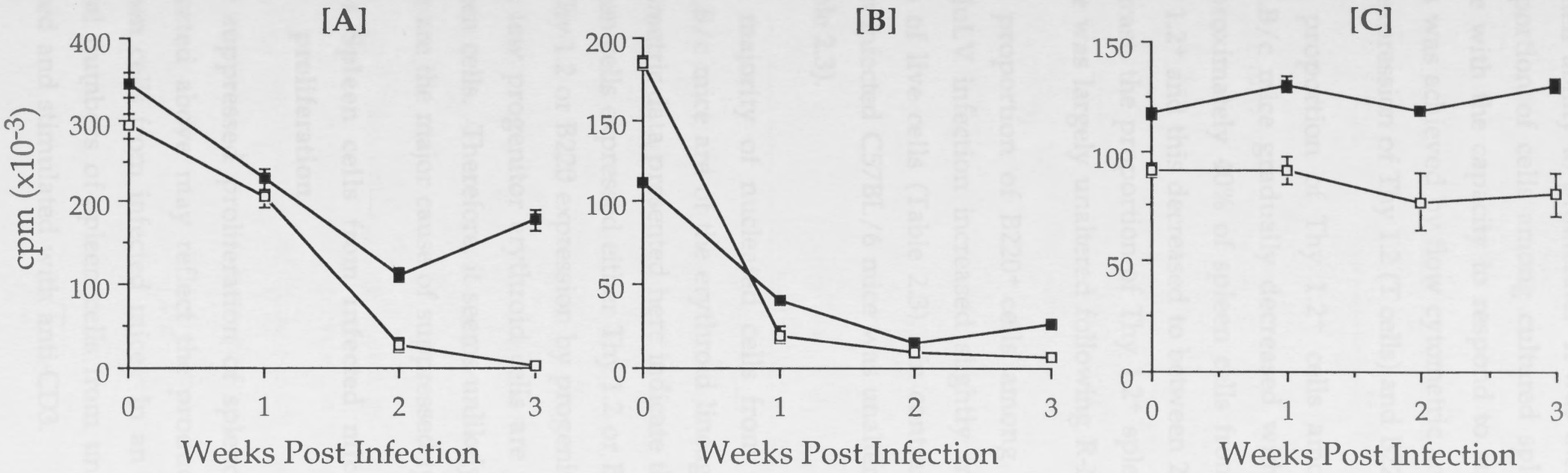


Figure 2.1 ***Proliferation of spleen cells and lymph node from R-MuLV infected mice***

Spleens and lymph nodes were removed from BALB/c (—□—) or C57BL/6 (—■—) mice which were either uninfected or infected with 10^4 pfu R-MuLV 1, 2 or 3 weeks previously. Single cell suspensions were prepared from pooled spleens or lymph nodes which were taken from mice at each time point. Cells were cultured with immobilised anti-CD3 antibodies or LPS for 72 h and labelled with 0.5 μCi [^{3}H]-TdR for a further 16 h. Data points indicate the mean cellular proliferation of 6 replicate culture (± SEM) as measured by [^{3}H]-TdR incorporation. **[A]** anti-CD3 stimulated spleen cells, **[B]** LPS stimulated spleen cells, **[C]** anti-CD3 stimulated lymph node cells. Data shown is representative of 3 experiments.

in their ability to proliferate. It was therefore necessary to determine the proportion of cells among cultured splenocytes from R-MuLV infected mice with the capacity to respond to stimulation with anti-CD3 or LPS. This was achieved by flow cytometric analysis of water lysed spleen cells for expression of Thy 1.2 (T cells) and B220 (B cells).

The proportion of Thy 1.2^+ cells among spleen cells prepared from BALB/c mice gradually decreased with time post-infection (Table 2.3). Approximately 40% of spleen cells from uninfected BALB/c mice were Thy 1.2^+ and this decreased to between 20-25% by 3 weeks p.i. In marked contrast, the proportion of Thy 1.2^+ spleen cells prepared from C57BL/6 mice was largely unaltered following R-MuLV infection (Table 2.3).

The proportion of $B220^+$ cells among BALB/c splenocytes following R-MuLV infection increased slightly from approximately 48% to over 60% of live cells (Table 2.3). In contrast, the proportion of $B220^+$ cells from infected C57BL/6 mice was unaltered during the course of infection (Table 2.3).

The majority of nucleated cells from the spleen of R-MuLV infected BALB/c mice are of the erythroid lineage (de Both *et al.*, 1978). The flow cytometric data presented here indicate that a majority of live, water lysed spleen cells expressed either Thy 1.2 or B220. There have been no reports of Thy 1.2 or B220 expression by progenitor erythroid cells which suggests that few progenitor erythroid cells are present after hypotonic shock of spleen cells. Therefore, it seems unlikely that reduced numbers of T or B cells are the major cause of suppressed lymphocyte proliferation.

2.3.4 Spleen cells from infected mice suppress normal spleen cell proliferation

The suppressed proliferation of splenocytes stimulated with anti-CD3 reported above may reflect the production of a suppressive factor by spleen cells from infected mice. In an attempt to resolve this question, equal numbers of spleen cells from uninfected and infected mice were mixed and stimulated with anti-CD3.

Table 2.3 ***Proportion of Thy 1.2$^+$ or B220$^+$ cells in the spleen of R-MuLV infected BALB/c or C57BL/6 mice***

Weeks Post Infection	% Thy 1$^+$ cells	% B220$^+$ cells
BALB/c		
Uninfected	38.8 ± 6.7	47.9 ± 1.4
1	34.3 ± 4.1	55.9 ± 9.6
2	28.8 ± 6.0	57.9 ± 9.2
3	23.9 ± 1.7[a]	62.7 ± 4.4[c]
C57BL/6		
uninfected	40.8 ± 6.7	57.3 ± 6.0
1	39.9 ± 2.7	54.4 ± 5.3
2	40.4 ± 6.6	56.9 ± 3.6
3	40.7 ± 8.9	54.3 ± 8.1

Groups of 4 BALB/c or C57BL/6 mice were infected with R-MuLV 1, 2 or 3 weeks previously. Single cell suspensions were made from pooled spleens in each group. The cells were subjected to hypotonic shock to remove red blood cells. 5-10x10^5 cells were stained with either biotinylated anti-Thy 1.2 mAb followed by FITC conjugated streptavidin or FITC conjugated anti-B220 mAb. The proportion of cells expressing Thy 1.2 or B220 was measured by flow cytometry. Forward and side scatter were used to gate for live cells. Results are expressed as the percentage of live cells positively stained for Thy 1 or B220 expression.

Data represents mean of three independent experiments ± SD

a) significantly less than uninfected mice $p=0.02$

b) significantly greater than uninfected mice $p<0.05$

c) significantly greater than uninfected mice $p<0.01$

Addition of cells from infected BALB/c mice did not alter (1 week p.i.) or markedly inhibited T cell proliferation (2 and 3 weeks p.i.; Table 2.4). Similarly, cells from C57BL/6 mice slightly increased (1 week p.i.) or suppressed (2 or 3 weeks p.i.; Table 2.4) proliferation of uninfected cells however, due to the range in the data this difference was not statistically significant ($p>0.05$, Table 2.4). As expected, addition of uninfected BALB/c or C57BL/6 spleen cells to an equal number of uninfected cells resulted in a large increase in proliferative responses to anti-CD3 stimulation.

2.3.5 Effect of T cell enrichment on anti-CD3 stimulated proliferation

The decrease in Thy 1.2^+ spleen cells observed in BALB/c mice infected with R-MuLV may account at least in part for the observed decrease in T cell proliferation. Virus infected progenitor erythroid cells and cell debris may also suppress T cell proliferation. To test these propositions, spleen cells from infected mice were enriched for Thy 1.2^+ cells in an attempt to obtain approximately equal numbers of Thy 1.2^+ cells among infected and uninfected spleen cell populations used in *in vitro* culture. To enrich for Thy 1.2^+ cells, a Percoll density gradient was used to remove contaminating low density erythroid progenitor cells (Faxvaag *et al.*, 1993), while B cells were removed using J11d antibody (Bruce *et al.*, 1981) plus complement mediated lysis (Section 2.2.8).

This enrichment protocol left a population of spleen cells from uninfected BALB/c mice that were approximately 60% Thy 1.2^+ (Table 2.5). The proportion of Thy 1.2^+ cells from spleen cells of infected BALB/c mice following enrichment was between 40 and 55%. Spleen cells from C57BL/6 mice which were enriched for Thy 1.2 expressing cells were approximately 70% Thy 1.2^+ (Table 2.5).

The proliferation of BALB/c T cell enriched spleen cells from infected mice was similar to that of T cell enriched splenocytes from uninfected mice (Table 2.6). This is in marked contrast to proliferation of whole splenocytes which was suppressed in infected mice. The enrichment of Thy 1.2^+ cells did not alter the suppressed proliferation of spleen cells from R-MuLV infected C57BL/6 mice (Table 2.6). The anti-CD3

Table 2.4 *Spleen cells from infected mice suppress uninfected spleen cell proliferation*

Mouse Strain	Uninfected Cells	Cells Added	Proliferation[a] (cpm x 10^{-3})
BALB/c	+	None	149.1 ± 14.2
	+	Uninfected	376.5 ± 7.7[b]
	+	1	165.8 ± 20.4
	+	2	83.3 ± 10.4[c]
	+	3	73.5 ± 20.5[d]
C57BL/6	+	None	61.5 ± 9.1
	+	Uninfected	98.0 ± 9.5[e]
	+	1	73.9 ± 10.8
	+	2	53.8 ± 9.8
	+	3	42.2 ± 4.5

$5x10^4$ spleen cells from uninfected mice were cultured alone or with $5x10^4$ spleen cells from uninfected mice or mice infected 1, 2 or 3 weeks earlier. Cells were stimulated with immobilised anti-CD3 mAb for 72 h. Proliferation was estimated by [^{3}H]-TdR incorporation for the next 18 h. Data shown is representative of 2 similar experiments.

a) Mean ± SEM proliferation of 4 wells

b) Significantly greater proliferation compared to $5x10^4$ uninfected cells ($p<0.001$)

c) Significantly less proliferation compared to $5x10^4$ uninfected cells ($p<0.01$)

d) Significantly less proliferation compared to $5x10^4$ uninfected cells ($p=0.02$)

e) Significantly greater proliferation compared to $5x10^4$ uninfected cells ($p=0.03$)

Table 2.5 ***Proportion of cells expressing Thy 1.2 in Thy 1.2$^+$ enriched spleen cells from R-MuLV infected BALB/c or C57BL/6 mice***

Strain of mouse	Weeks Post Infection	% Thy 1.2$^+$ cells
BALB/c	Uninfected	61.2 ± 4.0
	1	51.5 ± 3.4[a]
	2	41.5 ± 4.5[b]
	3	47.4 ± 2.9[b]
C57BL/6	uninfected	74.1 ± 8.6
	1	67.8 ± 1.5
	2	72.4 ± 7.5
	3	67.2 ± 11.2

Groups of 4 BALB/c or C57BL/6 mice were infected with R-MuLV 1, 2 or 3 weeks previously. Single cell suspensions were made from pooled spleens in each group. The cells were enriched for Thy 1.2$^+$ cells as described in section 2.2.13. 5-10x10^5 cells were stained with either biotinylated anti-Thy 1.2 mAb followed by FITC conjugated streptavidin. The proportion of cells expressing Thy 1.2 was measured by flow cytometry. Forward and side scatter were used to gate for live cells. Results are expressed as the percentage of live cells positively stained for Thy 1.2. Data represent the mean of three independent experiments ± SD.

a) significantly less than uninfected mice $p=0.03$

b) significantly less than uninfected mice $p<0.01$

Table 2.6 *Effect of T cell enrichment on spleen T cell proliferation*

	Proliferation (cpm $x10^{-3} \pm$ SEM)	
Weeks Post Infection	Whole Spleen	T Cell Enriched
BALB/c		
Uninfected	267.8 ± 6.8	216.9 ± 23.1
1	244.2 ± 4.6	271.3 ± 8.3
2	68.5 ± 11.7[a]	185.1 ± 12.3
3	9.7 ± 1.2[a]	184.1 ± 12.3
C57BL/6		
Uninfected	222.4 ± 19.8	291.5 ± 17.6
1	175.9 ± 7.7	307.1 ± 1.0
2	93.6 ± 6.3[a]	146.5 ± 8.8[a]
3	193.3 ± 6.6	134.7 ± 4.7[a]

Groups of 4 BALB/c or C57BL/6 mice were infected with R-MuLV 1, 2 or 3 weeks previously. Single cell suspensions were made from pooled spleens in each group. The cells were enriched for Thy 1.2^+ cells as described in section 2.2.13. $2x10^5$ whole spleen or Thy 1.2^+ enriched cells were stimulated with immobilised anti-CD3 antibodies for 72 h and labelled with 0.5 µCi $[^3H]$-TdR for a further 16 h. Data represents the mean ± SEM proliferation of 6 replicate cultures as indicated $[^3H]$-TdR incorporation. Results are representative of 2 similar experiments.

a) Significantly lower proliferation compared with proliferation by cells from uninfected mice ($p<0.001$)

stimulated proliferation of enriched splenocytes was similar to unfractionated spleen cells. These observations raise the possibility that suppression of BALB/c spleen T cell proliferation is due to suppressive factors and not to an intrinsic defect in the capacity of T cells to proliferate. In contrast, these findings are consistent with the impaired capacity of C57BL/6 T cells to proliferate being due to an intrinsic defect.

2.4 DISCUSSION

Initial experiments in this chapter were designed to examine the growth of R-MuLV in BALB/c and C57BL/6 mice. R-MuLV grew well in BALB/c mice with high levels of infectious virus and virus expressing cells found in spleen and high titres of infectious virus in plasma at all times examined. Levels of viral antigen in plasma increased with time following infection. In contrast, C57BL/6 mice were relatively resistant to R-MuLV infection. Infectious virus was not detected in plasma or cell free spleen homogenates, however virus expressing cells were present in the spleen at all times examined p.i. Peak numbers of virus producing cells were found at 7 days p.i. and declined thereafter. These data agree with numerous reports of R-MuLV replication in BALB/c and C57BL/6 mice. (Rauscher, 1962; Rauscher and Allen, 1964; Boiron *et al.*, 1965; Seidel and Lauenstein, 1969; Ishimoto *et al.*, 1971; McCoy *et al.*, 1972; Iwai *et al.*, 1994). Furthermore, the data in Tables 2.1 and 2.2 are similar to the reported growth of the closely related F-MuLV in these two strains of mice (Odaka and Yamamoto, 1962; Ceglowski *et al.*, 1974; Isaak *et al.*, 1979).

The degree of splenomegaly at 2-3 weeks p.i. is proportional to the dose of virus given (Chirigos, 1964). This is thought to be due to infection of progenitor erythroid cells and the subsequent mitogenic effect of R-SFFV upon these cells. Indeed, the titre of R-SFFV in a virus sample can be measured by counting the number of colonies of proliferating cells that appear in the spleen of infected mice at day 7-9 p.i. (Pluznik and Sachs, 1964). There have been many reports which use spleen weight (measured either by direct weighing or, for long term studies, palpation of the spleen in anaesthetised mice) to measure the efficacy of vaccine preparations, drug therapies or cytokine treatments in both R-MuLV and F-MuLV model systems (Johnson *et al.*, 1988; Chesebro, 1990; Hom *et al.*, 1991; Robertson *et al.*, 1992; Ruprecht *et al.*, 1992). In agreement with these reports, data in Table 2.1 and 2.2 show that spleen weight increases with time in R-MuLV infected BALB/c mice. Furthermore, virus antigen in plasma also increases with time p.i. In contrast, infectious virus in

plasma and spleen reach high levels early on in infection and remain so at all time points examined. As the spleen weight of R-MuLV infected C57BL/6 mice increased only slightly with time p.i. it may be argued that splenomegaly is not be a valid measure of R-MuLV induced disease in these mice. However, C57BL/6 nude mice or mice depleted of T cells (with anti-Thy 1.2 antibodies) developed significant splenomegaly following F-MuLV infection (Kitagawa *et al.*, 1986; van der Gaag and Axelrad, 1990; Kitagawa *et al.*, 1993). These observations suggest that in the absence of effective anti-R-MuLV immune responses C57BL/6 mice would also develop significant splenomegaly. Spleen weight was therefore used in some experiments to measure the outcome of R-MuLV infection in both BALB/c and C57BL/6 mice in Chapter 3 and 4.

The proliferation of splenic T cells from R-MuLV infected BALB/c mice was markedly suppressed. The proliferation of these cells was inhibited as early as 1 week p.i. and was almost completely ablated by week 2 p.i. These observations are similar to those of Häyry *et al.*, (1970) who showed that proliferation by PHA-stimulated spleen cells from infected mice was suppressed early during infection. The proliferation of splenic B cells from BALB/c mice was also markedly inhibited over the times examined. These observations are consistent with a number of reports of suppressed B cell function (antibody production) during R-MuLV infection of susceptible mice strains such as BALB/c (Ceglowski and Friedman, 1968; Millian and Schaeffer, 1968), SJL/J (Meredith *et al.*, 1978), DBA/2 (Tóth *et al.*, 1971b; Tóth *et al.*, 1971c).

C57BL/6 mice also exhibited immunosuppression following R-MuLV infection. The proliferation of both splenic T and B cells was found to be inhibited as early as 1 week p.i and was suppressed until 3 weeks p.i. The magnitude of suppressed T cell proliferation appeared to be greatest at week 2 p.i. while B cell proliferation was suppressed at all time points examined. Antibody production is also suppressed in R-MuLV infected C57BL/6 mice however the degree of suppression is lower than that found in susceptible mice (Borella, 1969; Seidel and Lauenstein, 1969).

In contrast with the suppressed proliferation exhibited by B cells from R-MuLV infected BALB/c mice, Meredith *et al.*, (1978) showed that LPS induced proliferation by spleen cells from highly susceptible SJL/J mice not suppressed following R-MuLV infection. In addition, R-MuLV infection of C57BL/10 mice was found to have little effect on proliferation of spleen T and B cells (Meredith *et al.*, 1978). The cause of the discrepancy between the data presented here and the findings of Meredith *et al.*, (1978) is unclear, however it may be due to genetic differences between the mice used here (BALB/c and C57BL/6) and those used by Meredith *et al.*, (1978; SJL/J and C57BL/10).

The proliferation of lymph node T cells from BALB/c and C57BL/6 mice was unaffected by R-MuLV infection. These findings are similar to those of Häyry *et al.*, (1970) who showed that R-MuLV infection of BALB/c mice had no effect on the proliferation of PHA stimulated lymph node cells. Furthermore, Gabrilovich *et al.*, (1993) showed that R-MuLV infection of BALB/c or C57BL/10 mice had little effect on the capacity of lymph node T cells to proliferate in response to allogeneic stimulation. The data presented here are also similar to those of Genovesi *et al.*, (1982), who showed that F-MuLV infection of susceptible DBA/2 mice had little effect on Con A stimulated proliferation of lymph node T cells.

The causes of suppressed lymphocyte proliferation in spleens of R-MuLV infected mice are unclear. As there is a large increase in the number of progenitor erythroid cells in the spleen of R-MuLV infected BALB/c mice it has been postulated that the suppressed proliferation may due to a decrease in the proportion of lymphoid cells in the spleen (Häyry *et al.*, 1970). Analysis of spleen cells by FACS showed that the proportion of cells expressing the T cell marker Thy 1.2^+ in R-MuLV infected BALB/c mice became progressively reduced with time p.i. While this reduction appears to be significant, it may not be sufficient to explain the near total suppression of T cell proliferation in these mice. Furthermore, proliferation by spleen T cells from C57BL/6 mice was suppressed without a reduction in the proportion of Thy 1.2^+ cells. Similarly, proliferation by spleen B cells from infected mice was impaired but the proportion of $B220^+$ cells in spleens of both strains of mice was unaltered

or only slightly increased following infection. Together, these data argue against changes in the proportion of splenic T or B cells as the predominant mechanism of immunosuppression.

Flow microfluorometry of splenocytes from infected BALB/c mice demonstrated that at least 90% of cells were Thy 1.2$^+$ or B220$^+$ cells (Table 2.3). Based on this finding it would appear that less than 10% of spleen cells used in culture were progenitor erythroid cells as neither Thy 1.2 or B220 markers have been shown to be expressed by progenitor erythroid cells. This conclusion is in marked contrast to previous reports which estimated that 3 weeks p.i. progenitor erythroid cells comprise over 60% of spleen cells in BALB/c mice (de Both *et al.*, 1978). On the data presented in this chapter it is difficult to satisfactorily explain this inconsistency. It may be that progenitor erythroid cells were vulnerable to lysis by exposure to hypotonic conditions which were used to lyse red blood cells prior to culture. May-Grünwald Giemsa staining of cytospin preparations of water lysed spleen cells would readily resolve this contention by showing the proportion of progenitor erythroid cells and lymphocytes following removal of red blood cells. At present it is not possible to determine the proportion of progenitor erythroid cells within the spleen by FACS analysis due to a lack of specific antibodies. To date the only antibody specific for erythroid cells is produced by the clone Ter 119 (Ikuta *et al.*, 1990). This antibody is specific for highly differentiated erythroid cells (normoblast stage; Ikuta *et al.*, 1990) but not BFU-e and CFU-e (which are major targets of R-MuLV; see Section 1.9.2) and would therefore appear to be of limited value here.

An alternative possible explanation of the discrepancy between the data described here and that by de Both *et al.*, (1978) is the coexpression of Thy 1 and B220 by spleen lymphocytes following R-MuLV infection. Snapper *et al.*, (1988) found that B cells stimulated with LPS or antigen in the presence of IL-4 were induced to express surface Thy 1. This hypothesis seems unlikely as cytokine expression analysis (see Chapter 3) indicated that IL-4 synthesis is not induced following R-MuLV infection, however at this time it is premature to dismiss this possibility. Further experiments designed to examine splenocyte expression of other T and B

cell markers such as CD3, CD4, CD8 and surface IgM during R-MuLV infection may clarify the proportion of lymphocytes in the spleen of R-MuLV infected mice.

Addition of spleen cells from infected BALB/c mice to was found to markedly suppress the proliferation of spleen T cells from uninfected mice. Spleen cells from R-MuLV infected C57BL/6 mice also inhibited the proliferation of spleen T cells from uninfected mice although this effect was not as pronounced. This effect was more pronounced when cells from mice infected 2 and 3 weeks earlier were used. These data indicate that an immunosuppressive factor was produced by spleen cells from infected mice. The identity of this suppressive factor was not characterised, however this is clearly an interesting finding which requires further study. The possible nature of this suppressive factor is discussed below.

Perhaps the most likely source of this suppression is R-MuLV itself. Mixing experiments similar to those described here have shown that F-MuLV infected spleen cells inhibit the ability of uninfected splenocytes to generate antibody producing cells (Kately *et al.*, 1974). The inhibition of proliferation was independent of cell contact and was blocked by anti-F-MuLV antibodies which suggests that the immunosuppressive factor was associated with F-MuLV. Similar experiments using anti-R-MuLV antibodies in the mixing experiments described in Section 2.3.4 would determine if R-MuLV was responsible for the observed suppression. Alternatively, addition of supernatants from cultured spleen cells of infected mice to uninfected cells with or without anti-R-MuLV antibodies would clarify the role of virus in suppressed T cell proliferation. If R-MuLV was found to be the suppressive factor described in Section 2.3.4, subsequent experiments may then determine whether infectious virus was required to mediate this effect. This may be achieved by inactivation (by heat or UV irradiation) of R-MuLV present in supernatants from cultures of cells from infected mice prior to addition to normal cells.

The suppressive effect reported in Section 2.3.4 may be mediated by virus encoded peptides. A number of investigators have demonstrated that purified, inactivated retrovirus virions (Orosz *et al.*, 1985) and some retroviral proteins such as p15E encoded by MuLV (Snyderman and Ciancolo, 1984; Ruegg *et al.*, 1989a) inhibit a range of immune responses. Moreover, disrupted R-MuLV particles were found to inhibit PHA induced proliferation by BALB/c spleen cells (Fowler *et al.*, 1977), while p15E protein of R-MuLV inhibits T cell proliferation *in vitro* (Ruegg *et al.*, 1989a). Addition of antibodies which bind specific viral proteins during mixing experiments may resolve their role in immunosuppression associated with R-MuLV infection.

The level of R-MuLV infection in each immune cell populations found within the spleen and lymph nodes of infected mice was not determined. This information is of interest as infection of immune cells by R-MuLV may alter their function leading to suppressed responses. Furthermore, this data may help to explain the correlation of the degree of immunosuppression associated with R-MuLV infection with the level of virus replication in BALB/c and C57BL/6 mice. Determination of virus levels and targets of virus infection in spleen and lymph node may also shed light on how R-MuLV infection markedly suppresses spleen but not lymph node T cells function.

In another attempt to clarify the nature of suppressed T cell proliferation, efforts were made to obtain purified population of T cells from the spleen cells from infected mice. Removal of low density cells, J11d$^+$ cells and debris from spleen cells of R-MuLV infected BALB/c mice restored T cell proliferation to levels similar to those of cells from uninfected mice treated in a similar manner. These data suggest that the suppressed T cell proliferation is not due to defects intrinsic to spleen T cells but is mediated by other factors. In contrast, similar treatment of spleen cells from R-MuLV infected C57BL/6 mice did not ameliorate the impaired T cell proliferation exhibited by untreated spleen cells. This observation raises the possibility that a different mechanism may be involved in suppression of C57BL/6 T cell proliferation. Clearly further investigation of the mechanisms of R-MuLV associated immunosuppression in

C57BL/6 mice is required before definitive statements can be made concerning this issue. Indeed, isolation of highly pure populations of immune cells eg. T cell subsets, B cells, macrophages would allow the function of each population to be assessed without the complicating presence of other cells.

The protocol employed to obtain similar levels of T cells in splenocyte populations from uninfected and infected mice was found to give relatively poor enrichment of T cells ie. 50-70%. It is unclear why these levels were so low. The protocol used was based on reports which showed that Percoll gradients could successfully separate T cells from progenitor erythroid cells in spleen cells from F-MuLV infected mice (Lopez-Cepero *et al.*, 1988; Faxvaag *et al.*, 1993). Furthermore treatment of splenocytes from normal or vaccinia infected mice with J11d antibody plus complement has been successfully used to remove B cells in our laboratory (Carpenter *et al.*, 1994). It is a possibility that the density of progenitor erythroid cells which accumulate during R-MuLV infection may be different to those found in F-MuLV infected mice, however there is no data to support this contention. In the light of the inefficiency of the enrichment protocols used here, alternative methods may have yielded better levels of purification. Positive selection of lymphocyte subpopulations using specific antibodies coupled to magnetic beads may be a more satisfactory method. An alternative approach would be to use fluorescence activated cell sorting of spleen cells to obtain relatively pure populations of cells.

Activated macrophages have been shown to inhibit lymphocyte proliferation in a wide range of infectious disease models including parasite infestations (Wellhausen and Mansfield, 1979) as well as bacterial (Al-Ramadi *et al.*, 1992) and viral infections (Rowland *et al.*, 1994; Pertile *et al.*, 1995). A number of soluble molecules produced by activated macrophages, such as NO, prostaglandins, H_2O_2 or the cytokine TGF-β have been shown to mediate these effects (Albina *et al.*, 1991). Clearly, activated macrophages are a potential source of immunosuppression associated with R-MuLV infection. The removal of macrophages from spleen cells of infected mice or the addition of activated macrophages to

splenocytes from normal mice may clarify the role of activated macrophages in the immunosuppression associated with R-MuLV infection. Alternatively, inhibition of the suppressive factors produced by activated macrophages may also clarify the importance of these molecules in R-MuLV induced immunosuppression. The role of two of these molecules (NO and prostaglandins) in R-MuLV immunosuppression is examined in Chapter 4.

Suppression of IL-2 production by T cells has been found in a number of retroviral infections such as F-MuLV (Lopez-Cepero *et al.*, 1988; Soldaini *et al.*, 1991), LP-BM5 MuLV (Gazzinelli *et al.*, 1992) and HIV (Murray *et al.*, 1984). As IL-2 acts as a growth factor for stimulated T cells (Swain, 1991), impaired IL-2 synthesis may be integral to the suppressed spleen T cell proliferation observed following R-MuLV infection. This hypothesis is examined in Chapter 3.

In summary, the results in this chapter show that the capacity of spleen but not lymph node lymphocytes to proliferate is suppressed following R-MuLV infection. This effect is more pronounced in susceptible BALB/c mice compared with resistant C57BL/6 mice. Analysis of lymphocyte populations in the spleen indicated that suppressed *in vitro* lymphocyte proliferation may not be due to changes in the proportions of different lymphocyte subgroups in the spleen.

Chapter 3

The role of cytokines and T cell subsets in resistance or susceptibility to R-MuLV

3.1 INTRODUCTION

The failure to control or resolve infectious disease often results from the generation of inappropriate immune responses, rather than the lack of immune responses (Bretscher *et al.*, 1992; Powrie and Coffman, 1993a). The observation that cytokine production by Th2 $CD4^+$ T cell clones can be cross-regulated by cytokines produced by Th1 clones (and vice versa) raised the possibility that *in vivo* CMI and humoral immune responses were regulated by cytokines (Mosmann *et al.*, 1986b; Mosmann and Coffman, 1989). Parasite infestation of inbred mice with differing susceptibilities to disease clearly demonstrated the role of cytokines in the generation of protective or non-protective immune responses (reviewed in Sher *et al.*, 1992). In the context of virus infections, the expression of strong CMI responses is often associated with the generation of protective immune responses (Blanden, 1974). This is well illustrated by pox virus infections of mice, where viral clearance is dependent on the generation of cytotoxic T cells (Blanden, 1971b; Blanden, 1971a) and the production of IFN-γ (Ruby and Ramshaw, 1991; Karupiah *et al.*, 1993a). In contrast, the expression of the type 2 factor, IL-4, by recombinant vaccinia viruses inhibits the generation of CTL and markedly delays clearance of infection (Andrew and Coupar, 1992; Sharma *et al.*, 1996).

The anti-R-MuLV immune response differs quite markedly between strains of inbred mice. BALB/c mice produce only small amounts of anti-R-MuLV antibodies following infection, while C57BL/6 mice generate high titres of R-MuLV specific antibodies (Tóth *et al.*, 1971c; McCoy *et al.*, 1972). In addition, BALB/c mice generate weak anti-R-MuLV CMI responses, while resistant C57BL/6 mice produce a relatively strong CMI response (McCoy *et al.*, 1972; Mortensen *et al.*, 1973). The failure of BALB/c mice to generate a protective anti-R-MuLV immune response does not reflect a lack of recognition of R-MuLV antigens, as mice are protected following administration of a variety of vaccine preparations (Fink and Rauscher, 1964; Peters *et al.*, 1975; Kelloff *et al.*, 1976). These observations suggest that the failure of BALB/c mice to control R-MuLV infection reflects the lack of a vigorous immune

response rather than the generation of an inappropriate immune response. As discussed above, the production of certain patterns of cytokines are associated with the generation of particular types of immune responses. The lack of markedly polarised anti-R-MuLV immune responses in resistant or susceptible mice make it unlikely that resistance or susceptibility to R-MuLV stems from the mutually exclusive production of type 1 or type 2 cytokines as found in other model systems (reviewed in Mosmann and Sad, 1996).

Nevertheless, the marked differences in anti-R-MuLV immune response generated by C57BL/6 and BALB/c mice may be a major determinant of the resistance or susceptibility exhibited by these strains. As cytokines are integral to the generation and regulation of immune responses, the expression and activities of cytokines and T cell subsets in resistant or susceptible strains infected with R-MuLV were investigated.

3.2 MATERIALS AND METHODS

3.2.1

Virus and infection of mice	As described in Section 2.2.2
Culture media	As described in Section 2.2.3
R-MuLV ELISA	As described in Section 2.2.7
Statistical analysis	As described in Section 2.2.15

3.2.2 Mice

BALB/c, C57BL/6, were used as described in Section 2.2.1. CBA/J and athymic nude Swiss mice were raised under specific pathogen-free conditions and were used at 8-12 weeks of age.

IFN-γ receptor gene knock out (IFN-γ $R^{o/o}$) and isogenic wild type (IFN-γ $R^{+/+}$) mice were obtained from Dr M. Aguet (Genentech, CA, USA) and generously provided by Dr A. Ramsay (Division of Cell Biology, JCSMR). Both strains were generated on a homozygous 129/Sv/Ev background (Huang *et al.*, 1993). 129/Sv/Ev mice have the genotype *H-2*^{bc}, *Fv-1*$^{nr/nr}$, *Fv-2*$^{s/s}$. Male mice were raised under specific pathogen free conditions and used at 8-10 weeks of age.

3.2.3 RNA extraction from spleen cells

RNA was extracted using the guanidinium thiocyanate/acid phenol extraction method (Chomczynski and Sacchi, 1987). Briefly, 2-5x10^7 freshly isolated spleen cells (Section 2.2.9) were collected, snap frozen in a dry ice ethanol bath and stored at -70°C until further processing. Frozen cells were denatured in guanidinium isothicyanate followed by extraction of DNA and proteins using acid phenol/chloroform. Following 2 rounds of isopropanol precipitation, the RNA was washed with ice cold 70% ethanol, dried and resuspended in diethyl pyrocarbonate (DEPC) treated water (Sambrook *et al.*, 1989). Total RNA was quantified spectrophotometrically using absorbance at 260nm. RNA integrity was

determined by electrophoresis of 3 µg total RNA through a denaturing, formaldehyde, 1% agarose gel (Sambrook *et al.*, 1989) followed by staining with ethidium bromide (10 µg/ml) for 20 min and destaining for at least 1 h. Intact RNA had a typical 2:1 ratio of 28S to 18S ribosomal RNA bands.

3.2.4 cDNA synthesis

1 µg of total RNA was diluted in 8 µl of DEPC treated water, denatured at 80°C for 5 min and then chilled on ice for 5 min. A master mix of cDNA synthesis reagents was prepared and added to the denatured RNA in a 12 µl volume. Each cDNA reaction had a final volume of 20 µl and contained 2 mM of dATP, dCTP, dGTP and dTTP (Pharmacia), 50 mM Tris-HCl pH 8.3 (at 42°C), 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM spermidine, 8 U AMV reverse transcriptase, 20 U RNasin and 0.5 µg oligo dT_{15} (Promega, Madison, WI, USA). The oligo dT was allowed to anneal at 23°C for 10 min followed by cDNA synthesis for 45 min at 42°C. The reaction was terminated by heating the reaction mixture at 95°C for 5 min.

3.2.5 PCR amplification of cDNA

PCR amplification was used to detect cDNA encoding cytokines and the 'housekeeping gene' hypoxanthine phosphoribosyl transferase (HPRT). Primer sequences were taken from previously published reports and synthesised by the Biomolecular Resources Facility of the JCSMR or purchased from commercial sources. Primers used for PCR are listed in Appendix 1. All primers were selected to span at least one intron to allow differentiation between amplification of cDNA and genomic DNA.

The cDNA prepared in Section 3.2.4 was diluted to a volume of 100 µl with water so as to have the equivalent of 50 ng of cDNA per 5 µl. Aliquots of 7 µl were stored at -20°C until amplification. A PCR master mix was prepared and 15 µl of the mix was added to 5 µl of cDNA. Each reaction contained 0.2 U Supertaq polymerase (Stehelin & CIE AG, Basel, Switzerland), 10 mM Tris-HCl pH 9 (25°C), 50 mM KCl, 0.01% gelatin, 1.5

mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM dATP, dCTP, dGTP and dTTP (Pharmacia) and 10 pMol of each appropriate forward and reverse primer.

PCR amplification of cDNA was conducted using a FTS-1 Capillary Thermal Sequencer (Corbett Research, Mortlake, NSW, Australia). After incubation for 3 min at 94°C the cDNA was amplified using the following conditions. Denaturation at 95°C for 5 sec, primer annealing at 55°C for 5 sec (IFN-γ, TNF-α, HPRT), 60°C for 5 sec (IL-2) or 53°C for 10 sec (IL-4) followed by primer extension at 72°C for 1 min. Between 20 and 35 cycles were used to amplify each cDNA.

RNA was derived from cells known to express desired genes and used as positive controls for reverse transcription and PCR amplification. 1 µg of RNA from each source was handled as described in Section 3.2.4 and 3.2.5. RNA extracted from the T cell cone HDK-1, was used as a positive control for IFN-γ and TNF-α analysis (Cher and Mosmann, 1987). The T cell clone D10, was used as source of RNA encoding IL-4 (Rojo and Janeway Jr, 1988). Both T cell clones were stimulated with Con A (10 µg/ml; Pharmacia) for 16 h prior to RNA extraction. HDK-1 and D10.G4.1 cells were generously provided by Dr P. Hodgkin (JCSMR) and maintained as described in (Hodgkin *et al.*, 1990). RNA from α-6310 cells transfected with an expression plasmid encoding IL-2 (Section 3.2.8) was used as a positive control for IL-2 expression.

PCR products were analysed by electrophoresis through 1.5% agarose/TAE gel and visualised by ethidium bromide staining and UV illumination (Sambrook *et al.*, 1989).

3.2.6 Recombinant cytokines

Recombinant standard murine TNF-α and IFN-γ were kindly provided by Boehringer Ingelheim, Vienna, Austria; TNF specific activity was 1.2×10^7 U/mg, IFN-γ specific activity was 1×10^7 U/mg. Recombinant standard murine IL-6 and IL-4 were prepared by F. Lee and R. Kastelein (DNAX, Palo Alto, CA, USA) and provided by Dr P. Hodgkin (JCSMR). The IL-6 preparation was calibrated against the International Standard reference preparation of recombinant human interleukin-6 (88/514;

National Institute of Biological Standards and Control, Herts, U.K.) and therefore all titres have been given in arbitrary units (AU). The specific activity of the 88/514 preparation was $5x10^6$ AU/mg using a bioassay which employed the EBV-transformed B cell lines CESS or SKW6-CL4. Recombinant standard IL-2 was purchased from Genzyme Diagnostics (MA, USA) and had a specific activity of 2.5×10^6 U/mg.

3.2.7 Antibodies

Hybridomas were used to generate a variety of antibodies. Hybridomas were grown in MLCM and kept in log phase by adjusting cell concentration to 10^5/ml every 2 days. Antibodies were either used as ascites or culture supernatants.

Culture supernatants containing antibodies were generated by culturing hybridoma cells for 5-7 days. Cells were removed from the exhausted media by centrifugation at 500g for 10 min. The antibody containing supernatant was filtered and stored at -20°C.

Swiss outbred mice were used to generate antibody containing ascites. These mice were primed for hybridoma growth 10-14 days previously by i.p. injection of 500 µl of pristane (Sigma) and injected with $5x10^6$ hybridoma cells. After a further 10-14 days, the mice were killed and the ascites removed. The ascites was allowed to clot at room temperature for 1 h and then clarified by centrifugation at 500g for 10 min. Some batches of ascites were extracted with CCl_2FCClF_2 (Sigma) to remove lipids. An equal volume of CCl_2FCClF_2 was added to ascites followed by gentle mixing. The two phases were separated by centrifugation at 500g for 5 min. The upper, aqueous phase was removed, aliquoted and stored at -20°C until required.

Cytokine neutralising mAb used in this study were derived from the following hybridomas: S4B6 (anti-IL-2, Rat IgG2a; Mosmann *et al.*, 1986a), 11B11 (anti-IL-4, rat IgG1; Ohara and Paul, 1985), 20F3 (Anti-IL-6, rat IgG1; Starnes Jr *et al.*, 1990), JES5-2A5 (anti-IL-10, rat IgG1; Abrams *et al.*, 1992), R4-6A2 (anti-IFN-γ, rat IgG1; Spitalny and Havell, 1984), XT22

(anti-TNF-α, rat IgG1; Abrams *et al.*, 1992), GL113 (anti-β-galactosidase, rat IgG1; kindly provided by Dr. J. Abrams, DNAX).

Mice were depleted of cells expressing CD4 or CD8 using the monoclonal antibodies produced by hybridomas GK1.5 (Dialynas *et al.*, 1983) or D9 (Fazekas de St Groth *et al.*, 1986) respectively. Clone GK1.5 produces rat IgG 2b antibody, while clone D9 secretes mouse IgG 2a antibody. Antibody produced by clone SFR8-B6 (anti human HLA-Bw6; rat IgG 2b; Radka *et al.*, 1982) was used as a control antibody in depletion experiments. Hybridomas were grown and were used to generate antibody containing ascites as described above.

The concentration of antibody in various preparations was determined using an antibody specific capture ELISA system as described by (Coffman and Carty, 1986). The ELISAs used unlabelled and biotinylated polyclonal rabbit antisera specific for mouse IgG2a and Rat IgG1, IgG2b antibodies (Southern Biotechnology Associates Inc, Alabama, USA). Each ELISA used 96 well, round bottomed plates (Dynatek) and were developed and measured as described for the R-MuLV specific ELISA in Section 2.2.7. Known amounts of purified antibody were used to determine the absorbance produced by given amounts of antibody. A curve of best fit was used to calculate the levels of antibody in ascites or culture supernatant. Purified mouse antibodies from the clones UPC 10 (IgG2a; Sigma) and purified rat antibodies from clones GL113 (IgG1) and 30-H12 (IgG2b; generously provided by Dr P Hodgkin, JCSMR) were used as standards .

3.2.8 Cells used in cytokine bioassays

The IL-6 responsive cell line, B9, a B-cell hybridoma cell line, (Aarden *et al.*, 1987) was obtained from Dr L.A. Aarden (University of Amsterdam, Amsterdam, The Netherlands). B9 cells were maintained in H16 media supplemented with 10% of a Con A supernatant source of IL-6. The IL-2 or IL-4 dependent cell line HT-2 (Watson, 1979) was obtained from Dr P. Hodgkin (JCSMR) and was maintained in MLCM containing 50

U/ml murine IL-2. The IL-2 was prepared as a supernatant from α-6310 cells as described below.

The Con A supernatant source of IL-6 was generated by stimulating CBA/H mouse spleen cells with Con A. Single cell suspensions were made from spleens aseptically removed from 15 CBA/H mice. After washing, these cells were resuspended in 200 ml H16 containing 1% FCS and 5 μg/ml Con A (Pharmacia) and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 3 days. Cells and debris were removed by centrifugation at 500g for 10 min. Con A was precipitated with α-methyl-D-mannoside (2 g/100 ml; Sigma) and collected at 11,000g for 10 min at 4°C. The supernatant was sterilised by filtration through a 0.22 μm membrane and stored at -70°C.

Recombinant IL-2 was generated using α-6310 cells stably transfected with a plasmid expressing the IL-2 gene (Karasuyama and Melchers, 1988). Cells were grown in MLCM containing 1 mg/ml geneticin (GIBCO BRL) and kept in log phase by adjusting cell concentration to 10^5/ml every 2 days. To obtain supernatants containing IL-2, cells were washed with MLCM and cultured in the absence of geneticin. After 48 h, the cells were removed by centrifugation and the supernatant was aliquoted and stored at -20°C.

3.2.9 Cytokine biological assays

3.2.9.1 IL-2 Bioassay

The HT-2 cell line was used to determine the concentration of IL-2 in culture supernatants. Supernatant samples and recombinant IL-2 standard were serially diluted 1:2 across a 96 well flat-bottomed plate (Nunc, Kamstrup, Denmark) in a volume of 50 μl. HT-2 cells were washed twice with MLCM containing no IL-2 and $2x10^3$ cells were added to each well in 50 μl of MLCM. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. Proliferation was quantified using [^{3}H]-TdR incorporation (1μCi/well; ICN Radiochemicals) during the last 4 h of culture and was measured using a 96 well automatic harvester and a betaplate liquid scintillation counter (Pharmacia). IL-2

titres in culture supernatants were determined by comparison of the dilution giving half maximal proliferation of HT-2 cells with that of a concurrently run standard recombinant murine IL-2. As HT-2 cells also proliferate in response IL-4, IL-2 specific proliferation was determined by neutralising IL-4 in culture supernatants with anti-IL-4 mAb (11B11) which was generously provided by Dr P. Hodgkin (JCSMR) and was used at 10 μg/ml.

3.2.9.2 IL-6 Bioassay

IL-6 was measured using the IL-6-responsive cell line, B9. A 50 μl sample of culture supernatant or recombinant human IL-6 was serially diluted in H16 medium across a 96 well flat bottomed plate (Nunc) and cultured with 10^4 washed B9 cells in a total volume of H16 media. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 44 h. Proliferation was determined by [^{3}H]-TdR incorporation over the last 4 h of culture as described in Section 3.2.9.1. The concentration of IL-6 supernatants was determined by comparison of the dilution producing half maximal proliferation of B9 cells with that of proliferation generated by standard recombinant human IL-6

3.2.10 Cytokine ELISAs

3.2.10.1 TNF-α and IFN-γ ELISA

TNF-α and IFN-γ in culture supernatants were measured by indirect ELISA methods (Sheehan *et al.*, 1989) and were performed as described in Carpenter *et al.*, (1994). Anti-IFN-γ and anti-TNF-α antibodies were generously provided by Dr E. Carpenter and Mrs S. Fordham (Division of Cell Biology, JCSMR). Briefly, the IFN-γ ELISA used protein A purified R4.6A2 hybridoma cell ascites fluid (American Type Culture Collection, Rockville, MD, USA), polyclonal rabbit anti-Mu-IFN-γ antisera and alkaline phosphatase conjugated sheep anti rabbit antisera (Silenus, Parkeville, Victoria, Australia). The TNF ELISA employed immobilised hamster anti-mouse TNF mAb, TN3-19.12, (Sheehan *et al.*, 1989; kindly provided by Celtech, Berkshire, U.K.) polyclonal rabbit anti-Mu-TNF-α antisera and alkaline phosphatase conjugated anti-rabbit Ig (Silenus).

Both ELISAs utilised 96 well, flat bottomed ELISA plates (Titertek, Flow Laboratories, The Netherlands). The developed ELISAs were measured using conditions described for the R-MuLV specific ELISA in Section 2.2.7. A standard amount of recombinant cytokine was used to determine optical densities for given amounts of IFN-γ or TNF-α. A curve of best fit was determined and used to calculate the levels of cytokine in culture supernatants. The lower limit of the assays were routinely found to be 0.3 ng/ml (IFN-γ) and 160 pg/ml (TNF-α)

3.2.10.2 IL-4 ELISA

IL-4 in culture supernatants was assayed using indirect capture ELISA. Antibody reagents were generously provided by Ms K. Doherty (Division of Cell Biology, JCSMR). The coating antibody (11B11, 5 µg/ml) was diluted in 15 mM Na_2CO_3, 35 mM $NaHCO_3$ coating buffer (pH 9.6) and was bound to 96 well, round bottomed ELISA plates (Dynatech) by incubation overnight at 4°C in 50 µl/well. Unbound 11B11 was removed by washing each well 3 times with phosphate-buffered saline (PBS) containing 0.1% Tween (Sigma; PBS-Tween). Protein binding sites in each well were blocked by the addition of 100 µl 3% BSA PBS-Tween followed by incubation for 1 h at 37°C. After washing three times in PBS-Tween, 50 µl of diluted sample or recombinant murine IL-4 was added to each well and allowed to bind for 2 h at room temperature. The plates were washed again and 50 µl of biotinylated anti-IL-4 antibody (BVD6; 625 ng/ml) was added to each well and allowed to bind for 1 h at room temperature. After washing, 50 µl of streptavidin conjugated-alkaline phosphatase (Amersham; diluted 1:1000) was added to each well and incubated for 1 h at room temperature. The plates were washed, developed and measured as described in Section 2.2.7. A standard curve was generated for each ELISA and a curve of best fit applied. The equation describing the curve was used to determine the cytokine levels in supernatant samples. The lower limit of the assay was routinely found to be 1.5 U/ml.

3.2.11 Preparation of spleen cell culture supernatants for cytokine assay

Supernatants from spleen or lymph node cells were produced by stimulation with plate bound anti-CD3 as described for proliferation assays (Section 2.2.10). Plates were centrifuged at 200g for 2 min prior to removing supernatants. Supernatants for IL-2 assay were taken after 24 h culture. After 72 h, supernatants were taken and frozen at -20°C until assayed for IFN-γ, IL-6, IL-4 or TNF-α content.

3.2.12 Removal of adherent spleen cells.

Adherent cells were removed from spleen cells (prepared in Section 2.2.9) by incubating $5x10^7$ cells at 10^7/ml in a 60 x 15 mm petri dish (Nunc) for 1 h at 37°C. Non-adherent cells were removed by washing the petri dish with warm MLCM and were allowed to adhere to a second petri dish for a further 30 min at 37°C. Non-adherent cells were again removed by gentle washing, collected by centrifugation at 200g for 5 min and resuspended in 5 ml MLCM. The cells were subsequently treated as being 10^7 cells/ml and used to produce culture supernatants as described in Section 3.2.11 Adherent cells were collected by scraping with a rubber policeman, washed and counted. The proportion of Thy 1.2^+ cells was determined using flow cytometry as described in Section 2.2.12 and 2.2.13.

3.2.13. *In vivo* neutralisation of cytokines during R-MuLV infection

Monoclonal antibodies which were specific for a given cytokine were used to neutralise cytokines *in vivo*. Groups of BALB/c and C57BL/6 mice were treated with ascites diluted in PBS to 2.5 mg/ml antibody and was given at 0.5 mg/dose in 200 µl i.p. Antibody was given on days -1, 1, 3, 7, 10 and every 3 days thereafter. Half the mice in each group were infected with 10^4 pfu R-MuLV i.p. on day 0. After 14 days, mice were sacrificed and spleen weight of each mouse was determined as an indicator of disease progression as described by (Chirigos, 1964).

3.2.14 R-MuLV infection of IFN-γ $R^{+/+}$ and IFN-γ $R^{o/o}$ mice

Groups of IFN-γ $R^{+/+}$ and IFN-γ $R^{o/o}$ mice were inoculated with 10^4 pfu R-MuLV. A number of parameters of virus growth were examined including spleen weight, viral antigen in plasma and *in vitro* proliferation of spleen T and B cells.

3.2.14.1 Virus growth

Blood was obtained from mice via tail vein bleeding and was placed in heparinised tubes (Section 2.2.8). Plasma was separated from blood cells by centrifugation at 12000g for 5 min. Plasma was isolated and stored at -20°C. Viral antigen in plasma was assayed by ELISA as described in Section 2.2.7

Spleens were taken from uninfected and infected mice and weighed. Half of each spleen was used to prepare single cell suspensions (Section 2.2.9) while the other half was fixed in 10% neutral buffered formalin prior to processing and embedding in paraffin. Serial, 4 µM sections were taken, stained with May-Grünwald Giemsa and mounted in mounting fluid.

3.2.14.2 Proliferation assays

Single cell suspensions were prepared from pooled spleens from BALB/c or C57BL/6 mice as described in Section 2.2.9. Spleen B and T cell proliferation was assayed as described in Section 2.2.10.

3.2.15 *In vivo* depletion of cells expressing CD4 or CD8

Groups of BALB/c and C57BL/6 mice were depleted of cells expressing CD4 and/or CD8 using GK 1.5 (anti-CD4) or D9 (anti-CD8) ascites. As a control, ascites from SRF8-B6 (anti-human HLA-Bw6) was used. Appropriate antibody was diluted to 2.5 mg/ml antibody with PBS and given on days -1, 1, 3, 7, 10 and every 4 days thereafter. Half the mice from each group were infected with R-MuLV i.p. on day 0. After 21 days, mice were sacrificed and spleen weight of each mouse was determined as an indicator of disease progression as described by (Chirigos, 1964). Depletion of the appropriate cell type was assayed using fluorescence

microfluorometry as described in Section 3.2.16. Depletion resulted in loss of >90% of target cells from lymph nodes.

3.2.16 Immunofluorescence staining and flow cytometry

The efficiency with which CD4 or CD8 expressing cells were depleted was assayed using flow cytometry. The following mAb and antisera were used to determine the proportion of cells expressing specific cell surface antigens. Anti-CD4 , clone RL 172.4 (rat IgM; Ceridig *et al.*, 1985) and anti-CD8, clone 31M (rat IgG; Sarmiento *et al.*, 1980) were used as hybridoma supernatants and were generously provided by Dr J. Ruby (JCSMR). The supernatants were used undiluted. Rabbit polyclonal anti-rat κ chain (Immunotech S.A., Marseille, France) was used to detect cells which had bound either of the two rat mAb and was used at a dilution of 1:40 in PBS.

At the end of each depletion experiment the efficacy of cell depletion was determined by flow microfluorometry of single cell suspensions prepared from pooled brachial and inguinal lymph nodes (CD4 and CD8) from 2-3 mice. Staining of lymph node or spleen cells was performed on 10^6 cells using reagents described above (CD4, CD8). Cells were stained as described in Section 2.2.12 and analysed by flow cytometry as described in Section 2.2.13.

3.3 RESULTS

3.3.1 Qualitative analysis of cytokine mRNA expression in spleens of R-MuLV infected mice

Cytokines are integral to the generation and mediation of adaptive immune responses. It was therefore of interest to determine cytokine expression during R-MuLV infection. The expression of mRNA encoding IL-2, IL-4, IFN-γ, TNF-α and the 'housekeeping' gene HPRT in spleen cells during R-MuLV infection was examined by reverse transcriptase-PCR (RT-PCR). Cytokine expression was determined in both BALB/c and C57BL/6 mice at various stages of infection.

Figure 3.1 illustrates the expression of mRNA encoding IL-2 or IL-4 in the spleens of R-MuLV infected mice. R-MuLV infection of either BALB/c or C57BL/6 mice led to a slight reduction of IL-2 mRNA expression in the spleen. In BALB/c mice the effect was most marked at 21 days p.i. while in C57BL/6 mice expression was reduced at day 14 and 21. In contrast, IL-4 expression was largely unaltered following R-MuLV infection of mice of either strain.

The expression of IFN-γ and TNF-α mRNA by spleen cells was also examined (Figure 3.2). Production of IFN-γ mRNA was induced at low levels following R-MuLV infection of BALB/c mice and peaked at 2 weeks p.i. Expression of IFN-γ mRNA in spleen cells from infected C57BL/6 mice were also slightly elevated with levels being similar at all time p.i. examined. Slightly elevated levels of TNF-α mRNA were also found in the spleens of infected BALB/c mice, with peak levels observed at 2 weeks p.i. In contrast, R-MuLV TNF-α expression in the spleen cells from C57BL/6 mice was reduced at 2 weeks p.i.

The consistency of the RT-PCR procedure was demonstrated by RT-PCR amplification of HPRT mRNA (Figure 3.1) and equivalent ethidium bromide staining of 3 μg total RNA run through a formaldehyde gel (Figure 3.1).

Figure 3.1 ***Cytokine and HPRT mRNA expression by spleen cells from R-MuLV infected BALB/c and C57BL/6 mice***

Total RNA was extracted from spleen cells taken from BALB/c or C57BL/6 mice which were either uninfected or infected with R-MuLV 1, 2 or 3 weeks earlier. cDNA, equivalent to 50 ng of total RNA, was amplified by PCR through the number of cycles indicated (cycles) using cytokine specific primers. PCR products were run through 1.5 % agarose gels and stained with ethidium bromide. To confirm RNA integrity, 3 mg of total RNA was run through a 1% agarose-formaldehyde gel and stained with ethidium bromide. Data shown is representative of 2 batches of RNA and at least 1 cDNA synthesis and PCR amplification from each batch of RNA.

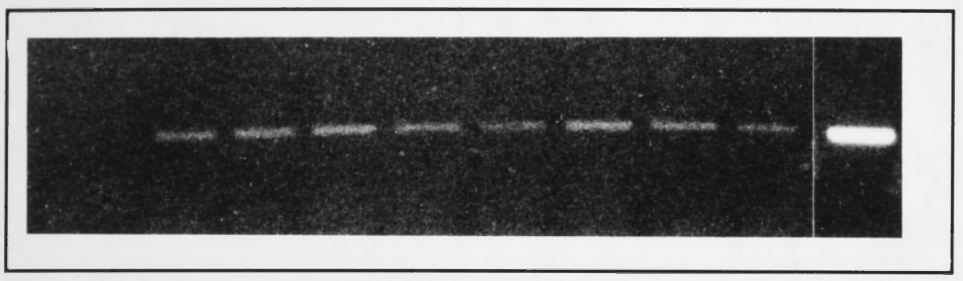
BALB/c C57BL/6

Cytokine Primers

- 0 1 2 3 0 1 2 3 +

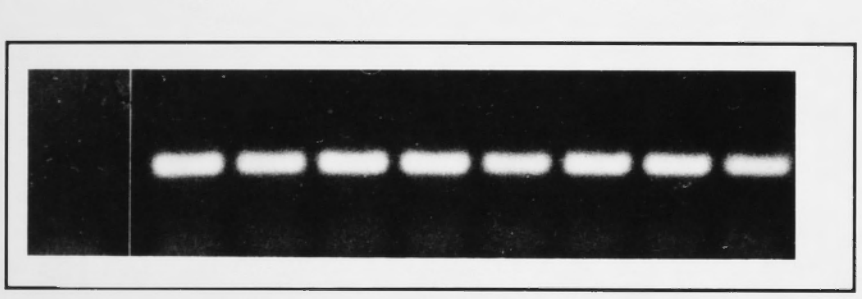
IL-2 (32)

- 0 1 2 3 0 1 2 3 +



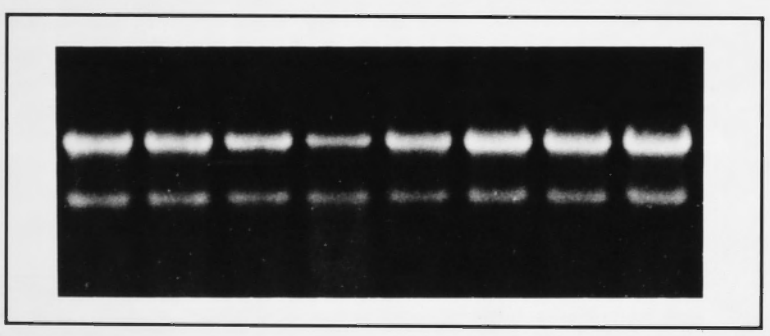
IL-4 (30)

- 0 1 2 3 0 1 2 3



HPRT (23)

0 1 2 3 0 1 2 3



TOTAL RNA

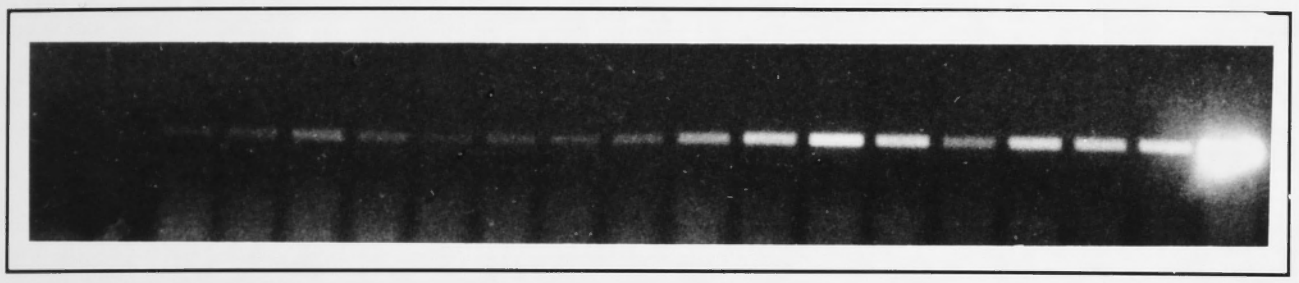
Figure 3.2 ***RT-PCR analysis of IFN-γ and TNF-α mRNA***

RNA was extracted from spleen cells of BALB/c and C57BL/6 mice which were uninfected or infected with R-MuLV 1, 2 or 3 weeks earlier. mRNA encoding IFN-γ or TNF-α was detected by RT-PCR amplification. RT-PCR of cDNA equivalent to 50 ng of total RNA was amplified through 22 or 25 cycles of PCR. PCR products were electrophoresed through 1.5% agarose and stained with ethidium bromide. Data shown is representative of 2 batches of total RNA and at least 1 cDNA/PCR amplification from each batch of RNA.

22 cycles **25 cycles**

BALB/c C57BL/6 BALB/c C57BL/6

\- 0 1 2 3 0 1 2 3 0 1 2 3 0 1 2 3 +

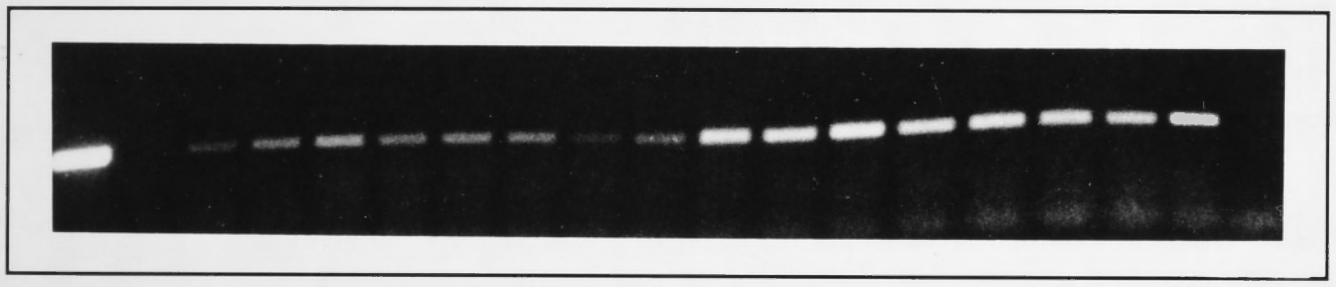


IFN-γ

22 cycles **25 cycles**

BALB/c C57BL/6 BALB/c C57BL/6

\+ 0 1 2 3 0 1 2 3 0 1 2 3 0 1 2 3 -



TNF-α

To demonstrate that the amplification of cytokine cDNA was proportional to the amount of input cDNA and to the number of cycles of amplification, serial 10 fold dilutions of cDNA known to contain cytokine sequences were amplified through 25, 30 and 35 cycles of PCR. The data shown in Figure 3.3 clearly demonstrates that by increasing the number of cycles of amplification low levels of cytokine cDNA (equivalent to 5 pg total RNA) were readily detected. The data also shows that amplification of high levels of input cytokine cDNA (equivalent 5 ng total RNA) rapidly becomes non-proportional and yields no further increases in amplified DNA.

3.3.2 Production of cytokines by spleen cells following *in vitro* stimulation with anti-CD3 antibodies

Next, the capacity of spleen cells from R-MuLV infected mice to produce cytokines was examined as it was important to confirm that changes in cytokine mRNA expression resulted in altered cytokine synthesis. Spleen cells were isolated, stimulated with immobilised anti-CD3 antibodies (anti-CD3) and the accumulation of cytokines in culture supernatants was assayed.

3.3.2.1 Effect of R-MuLV infection on IFN-γ production by spleen cells

Anti-CD3 stimulated spleen cells from uninfected mice produced substantial levels of IFN-γ. Supernatants collected from stimulated C57BL/6 cells consistently contained more IFN-γ than from BALB/c mice ($p<0.01$; Table 3.1). Anti-CD3 stimulated spleen cells from infected C57BL/6 mice produced 2-3 fold less IFN-γ compared to levels produced by cells from uninfected mice. BALB/c spleen cells initially produced increased levels of IFN-γ, while IFN-γ production was markedly suppressed ($p<0.01$) at 3 weeks p.i. Supernatants from unstimulated spleen cells from uninfected or infected mice contained less than 1 ng/ml IFN-γ (data not shown).

Figure 3.3 ***Qualitative RT-PCR analysis of cytokine mRNA expression by cytokine expressing cell line***

cDNA was prepared from total RNA derived from cytokine producing cell lines. Serial 10 fold dilutions of cDNA, equivalent to between 5 ng and 5 pg of total RNA were amplified through 25, 30 or 35 cycles of PCR using cytokine specific primers. PCR products were run through 1.5% agarose gels and stained with ethidium bromide.

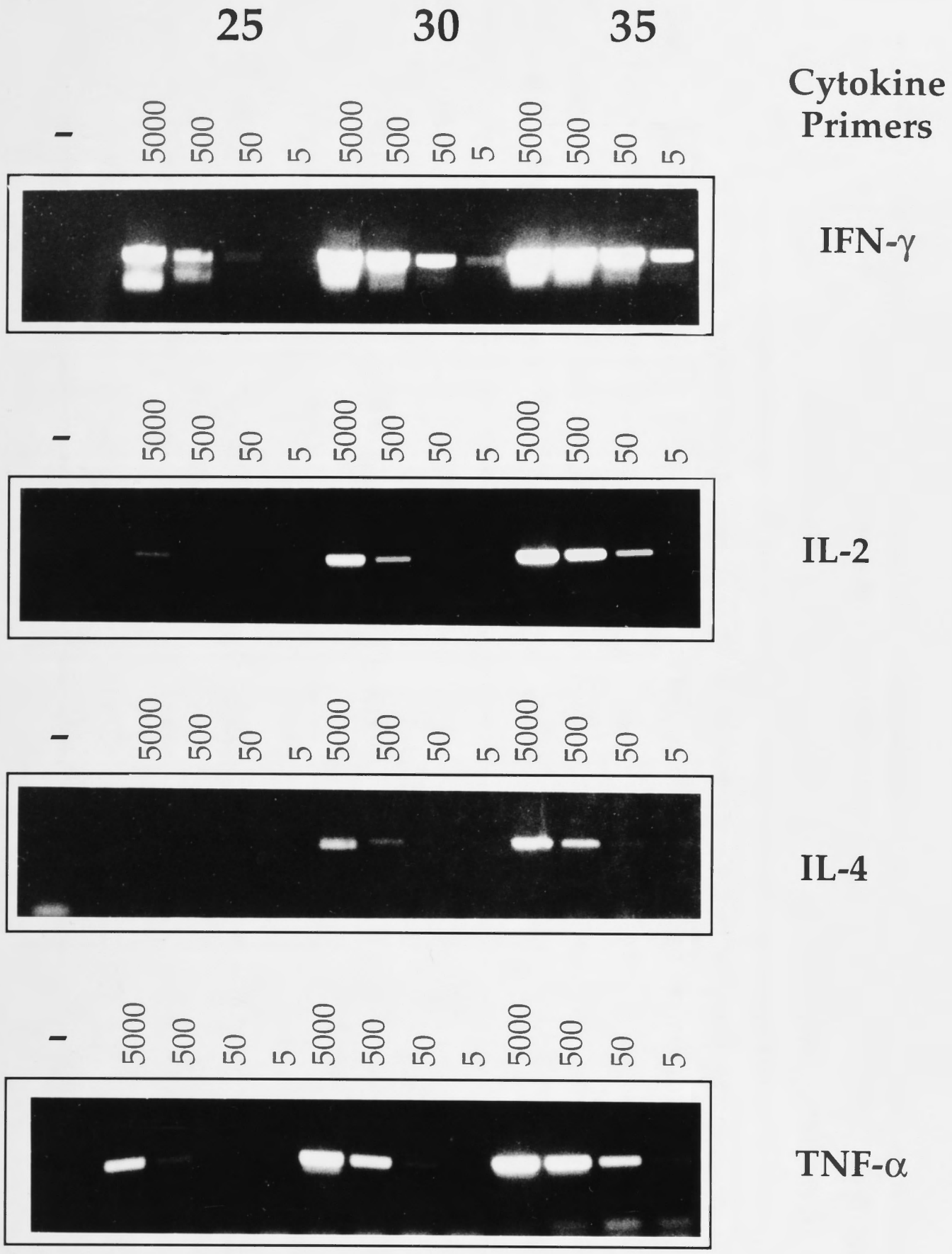

25
30
35
Cytokine Primers
- 5000 500 50 5 5000 500 50 5 5000 500 50 5
IFN-γ
- 5000 500 50 5 5000 500 50 5 5000 500 50 5
IL-2
- 5000 500 50 5 5000 500 50 5 5000 500 50 5
IL-4
- 5000 500 50 5 5000 500 50 5 5000 500 50 5
TNF-α

Table 3.1 Cytokine production by anti-CD3 stimulated spleen cells

	IFN-γ (ng/ml)		IL-2 (U/ml)		IL-6 (AU/ml)	
Weeks Post Infection	BALB/c	C57BL/6	BALB/c	C57BL/6	BALB/c	C57BL/6
Uninfected	17.1 ± 2.3	139 ± 18.0	2.36 ± 0.17	1.90 ± 0.23	3.53 ± 0.75	5.34± 0.48
1	57.1 ± 2.2[b]	84.2 ± 10.5[d]	3.15 ± 0.40[b]	1.96 ± 0.27	4.65 ± 0.90[a]	5.29 ± 1.07
2	12.8 ± 2.1[c]	58.7 ± 8.0[d]	1.07 ± 0.16[d]	1.69 ± 0.46	4.95 ± 0.85[a]	19.3 ± 2.16[b]
3	2.3 ± 0.2[d]	50.7 ± 5.3[d]	<0.5[d]	1.23 ± 0.27[c]	2.02 ± 0.21[b]	9.22 ± 0.27[b]

Spleen cells from R-MuLV infected or uninfected BALB/c or C57BL/6 mice were stimulated with anti-CD3 antibody *in vitro*. After 72 h culture, supernatants were taken and assayed for cytokine content. Cytokine levels are expressed as mean of 3 wells assayed in duplicate (± SD) and are representative of 2 (IL-2) or 3 (IFN-γ, IL-6) experiments. Cytokine levels found in supernatants from unstimulated cultures were as follows: IFN-γ <1 ng/ml ; IL-2 <0.5 U/ml ; IL-6 <0.25 AU/ml

a) Significantly greater ($p<0.05$) than levels in supernatants from cells of uninfected mice
b) Significantly greater ($p<0.001$) than levels in supernatants from cells of uninfected mice
c) Significantly less ($p<0.05$) than levels in supernatants from cells of uninfected mice
d) Significantly less ($p<0.001$) than levels in supernatants from cells of uninfected mice

3.3.2.2 IL-2 production by spleen cells from R-MuLV mice

Spleen cells derived from uninfected mice produced low levels of IL-2 following stimulation with anti-CD3. Supernatants from stimulated BALB/c splenocytes at 1 or 2 weeks p.i. were largely similar to levels produced by cells from uninfected mice (Table 3.1). In marked contrast, IL-2 was not detected in the supernatants from spleen cells at 3 weeks. The levels of IL-2 in supernatants from stimulated C57BL/6 spleen cells were largely unaltered at 1, 2 and 3 weeks p.i. (Table 3.1). IL-2 levels in supernatants from unstimulated spleen cell cultures were below the levels of detection in the HT-2 assay ie. less than 0.5 U/ml (data not shown).

3.3.2.3 Production of IL-6 by spleen cells from R-MuLV infected mice

IL-6 was also found in supernatants of spleen cells stimulated with anti-CD3. Cells from uninfected mice of either strain produced approximately the same levels of IL-6 (Table 3.1). Spleen cells from C57BL/6 mice initially produced unaltered levels of IL-6, however at 2 weeks p.i. IL-6 production was at markedly greater levels compared to production by cells from uninfected mice ($p<0.001$). At 3 weeks, IL-6 levels were still elevated over uninfected controls but significantly less than at week 2 p.i. ($p<0.001$). In contrast, IL-6 production by spleen cells from BALB/c mice was largely unaltered following R-MuLV infection. Unstimulated spleen cells also produced IL-6 but was at levels less than 10% of those found in stimulated cultures (data not shown).

Carpenter *et al.*, (1994) demonstrated that IL-6 production by spleen cells from vaccinia virus infected mice was independent of T cells but dependent on adherent cells. The role of adherent cells in IL-6 production by spleen cells from R-MuLV infected mice was therefore examined. Results in Table 3.2 show that adherent cells were required for peak IL-6 production by spleen cells from uninfected mice of either strain. Adherent cells comprised less than 2% of cells applied to the petri dishes. Flow cytometry of adherent cells demonstrated that less than 20%

Table 3.2 *IL-6 production by anti-CD3 stimulated non-adherent spleen cells*

Mouse	R-MuLV	Spleen cells	IL-6 (AU/ml)
BALB/c	-	Whole	2.03 ± 0.33
	-	Non-adherent	1.12 ± 0.13[a]
	+	Whole	1.96 ± 0.90
	+	Non-adherent	1.07 ± 0.35[b]
C57BL/6	-	Whole	0.91 ± 0.30
	-	Non-adherent	0.52 ± 0.05[a]
	+	Whole	5.31 ± 0.75
	+	Non-adherent	2.81 ± 0.90[a]

Whole or non-adherent spleen cells from uninfected mice or mice infected with R-MuLV 14 days earlier were stimulated with anti-CD3 antibodies. After 72 h culture supernatants were taken from 3 wells and assayed for cytokine content using the B9/IL-6 bioassay. Data expressed as mean IL-6 concentration of 3 wells assayed in duplicate (± SD). Data representative of two experiments. IL-6 levels found in supernatants from unstimulated cultures were less than 0.25 AU/ml.

a) $p<0.01$ versus whole spleen

b) $p<0.05$ versus whole spleen

expressed Thy 1.2 which suggests that the majority of these cells were not T cells and were presumably macrophages and monocytes (Mosier, 1984).

3.3.2.4 Cytokine production by anti-CD3 stimulated lymph node cells from R-MuLV infected mice

It was of interest to examine cytokine expression by lymph node cells since R-MuLV infection has markedly different effects on the capacity of spleen and lymph node cells to proliferate following stimulation with anti-CD3 (chapter 2). At various times p.i., cells from systemic lymph nodes were obtained, stimulated with immobilised anti-CD3 and the level of cytokines in culture supernatants were determined.

Lymph node cells derived from uninfected C57BL/6 and BALB/c mice produced significant levels of IFN-γ following stimulation with anti-CD3. Levels of IFN-γ produced by BALB/c lymph node cells were largely unaltered at 7 days p.i. (Table 3.3). At 14 and 21 days p.i. IFN-γ production was slightly elevated compared to uninfected mice. Lymph node cell culture supernatants from R-MuLV infected C57BL/6 mice also contained elevated amounts of IFN-γ compared to uninfected controls with peak production occurring at 14 days p.i. (Table 3.3). Culture supernatants from unstimulated lymph node cells contained levels of IFN-γ which were below limits of detection (0.3 ng/ml; data not shown).

Anti-CD3 stimulated lymph node cells from R-MuLV infected mice produced elevated amounts of IL-6 compared to uninfected controls (Table 3.3). Cultures of cells from BALB/c mice produced increasing amounts of IL-6 with time p.i. which reached peak levels at 21 days p.i. In a pattern similar to IL-6 production by anti-CD3 stimulated spleen cells, IL-6 production by C57BL/6 cells reached peak levels at 14 days p.i. and was still elevated 21 days p.i. although at concentrations less than 14 days. Levels of IL-6 in supernatants of unstimulated cultures were below limits of detection (<0.1 AU/ml; data not shown).

Table 3.3 Interferon-γ and interleukin-6 production by anti-CD3 stimulated lymph node cells

	Interferon-γ (U/ml)		Interleukin-6 (AU/ml)	
Weeks Post Infection	BALB/c	C57BL/6	BALB/c	C57BL/6
Uninfected	14.9 ± 1.3	5.8 ± 0.7	0.12 ± 0.01	0.13 ± 0.02
1	10.3 ± 0.4[c]	9.1 ± 0.6[a]	0.37 ± 0.04[b]	1.46 ± 0.09[b]
2	23.8 ± 1.2[a]	38.3 ± 6.8[b]	0.78 ± 0.06[b]	2.16 ± 0.32[b]
3	24.9 ± 1.5[a]	21.1 ± 1.2[a]	1.84 ± 0.29[b]	1.26 ± 0.30[b]

Lymph node cells from R-MuLV infected or uninfected BALB/c or C57BL/6 mice were stimulated with anti-CD3 antibody *in vitro*. After 72 h culture, supernatants were taken and assayed for cytokine content. Data expressed as mean cytokine concentration of 3 wells assayed in duplicate (± SD) and are representative of 2 experiments. Cytokine levels found in supernatants from unstimulated cultures were as follows: IFN-γ <1 ng/ml ; IL-6 <0.1 AU/ml

a) Significantly greater ($p<0.001$) than levels in supernatants from cells of uninfected mice

b) Significantly greater ($p<0.001$, Welch's *t* test) than levels in supernatants from cells of uninfected mice

c) Significantly less ($p<0.001$) than levels in supernatants from cells of uninfected mice

IL-2 was not detected in culture supernatants of anti-CD3 stimulated lymph node cells (data not shown). The limit of detection of the IL-2 assay used was 0.5 U/ml. IL-2 was not detected in supernatants from cultured cells from uninfected or infected BALB/c or C57BL/6 mice.

3.3.2.6 Production of IL-4 and TNF-α by immune cells from R-MuLV infected mice

Culture supernatants from anti-CD3 stimulated spleen and lymph node cells were also assayed for IL-4 and TNF-α. Stimulated spleen cells from uninfected BALB/c mice produced detectable levels of IL-4 (approximately 10 U/ml). In contrast, IL-4 was not detected in supernatants from spleen cells of infected BALB/c mice. IL-4 was not found in culture supernatants from uninfected or infected C57BL/6 mice (data not shown). In addition, IL-4 was not found in supernatants from anti-CD3 stimulated cultures of lymph node cells from BALB/c or C57BL/6 mice (data not shown). TNF-α was not detected in culture supernatants of stimulated spleen or lymph node cells from either strain of mice (data not shown).

3.3.3 *In vivo* neutralisation of cytokines with monoclonal antibodies

So far in this chapter, experiments have been designed to examine cytokine expression during R-MuLV infection. In order to demonstrate a functional role for these cytokines in resistance or susceptibility to R-MuLV, mice were treated with cytokine neutralising mAb.

Groups of BALB/c or C57BL/6 mice were treated with anti-cytokine mAb on days -1, 1, 3, 7 and 10 p.i. Half the mice in each group were infected with R-MuLV on day 0. Spleen weight on day 14 was used as a measure of disease progression and virus growth (Chirigos, 1964).

The treatment of R-MuLV infected BALB/c or C57BL/6 mice with neutralising antibodies to the cytokines IL-2, IL-4, IL-6 or to an irrelevant antigen had no significant effect on R-MuLV induced increases in spleen weight after 14 days (Table 3.4). Although there was an increase in mean spleen weights of BALB/c mice treated with anti-IL-6 mAb this was not

Table 3.4 In vivo *neutralisation of cytokines with mAb during R-MuLV infection*

Cytokine Neutralising Antibody	R-MuLV	Spleen weight (mg)
BALB/c		
None	-	99 ± 7
Anti-β-Gal	-	101 5
Anti-IL-2	-	104 ± 10
Anti-IL-4	-	110 ± 11
Anti-IL-6	-	87 ± 4
None	+	300 ± 10
Anti-β-Gal	+	291 ± 18
Anti-IL-2	+	318 ± 31
Anti-IL-4	+	279 ± 20
Anti-IL-6	+	456 ± 70
C57BL/6		
None	-	92 ± 7
Anti-β-Gal	-	77 ± 2
Anti-IL-2	-	73 ± 8
Anti-IL-4	-	78 ± 5
Anti-IL-6	-	75 ± 4
None	+	178 ± 14
Anti-β-Gal	+	233 ± 24
Anti-IL-2	+	165 ± 14
Anti-IL-4	+	167 ± 17
Anti-IL-6	+	188 ± 18

Groups of 8 BALB/c or C57BL/6 mice were treated with PBS or mAb to β-galactosidase, IL-2, IL-4 or IL-6 on days -1, 1, 3, 7 and 10 p.i. Each dose was 0.5 mg of antibody in ascites diluted in PBS and given i.p. Half the mice from each group were infected with 10^4 pfu R-MuLV i.p. on day 0 while uninfected mice received PBS. After 2 weeks, the spleen was taken from each mouse and weighed in tared tubes. Data expressed as mean spleen weight of 4 mice ± SEM.

significant (p=0.06). Neutralisation of TNF or IFN-γ also had no significant effect on R-MuLV induced splenomegaly in either strain of mouse (Table 3.5). The slightly increased mean spleen weight of anti-TNF-α mAb treated C57BL/6 mice (Table 3.5) was not statistically significant (p=0.08) nor was it reproducible in subsequent experiments (Table 3.6).

TNF-α and IFN-γ have been shown to synergise in direct antiviral activity and activation of cells such as macrophages (Wong and Goeddel, 1986; Ding *et al.*, 1988). In order to examine the role of these two factors in resistance to R-MuLV infection, anti-TNF-α and anti-IFN-γ mAb were administered to mice together during R MuLV infection. Data in Table 3.6 indicate that treatment with mAb against IFN-γ or TNF-α either alone or together had no effect on R-MuLV induced splenomegaly.

3.3.4 R-MuLV infection of IFN-γ $R^{o/o}$ and IFN-γ $R^{+/+}$ mice

Selective inactivation of the IFN-γ or IFN-γ receptor gene in mice has been shown to markedly alter the immune response to viruses and other pathogens (Dalton *et al.*, 1993; Huang *et al.*, 1993; Müller *et al.*, 1994). Mice with the IFN-γ receptor gene disrupted were used to further investigate the role of IFN-γ in the immune response during R-MuLV infection. The growth of R-MuLV in IFN-γ $R^{+/+}$ and IFN-γ $R^{o/o}$ mice as well as the ability of immune cells to proliferate *in vitro* was determined.

3.3.4.1 Growth of R-MuLV in mice lacking functional IFN-γ receptor

Groups of IFN-γ $R^{o/o}$ or IFN-γ $R^{+/+}$ mice were infected with R-MuLV and spleen weight and plasma viral antigen were determined as a measure of virus growth at 1, 2 and 3 weeks p.i.

Infection of IFN-γ $R^{o/o}$ mice led to an increase in mean spleen weight which was approximately 4 times that of normal spleens at 1 week p.i. Splenomegaly persisted at 2 and 3 weeks, but at lower levels than at 1 week (Table 3.7). In contrast, elevated spleen weights were not found in IFN-γ $R^{+/+}$ mice until week 3 p.i. At this time there was a marked

Table 3.5 In vivo *neutralisation of cytokines with mAb during R-MuLV infection*

Cytokine Neutralising Antibody	R-MuLV	Spleen weight (mg)
BALB/c		
None	-	117 ± 4
Anti-TNF-α	-	111 ± 6
Anti-IFN-γ	-	109 ± 3
Anti-IL-10	-	107 ± 5
None	+	408 ± 32
Anti-TNF-α	+	457 ± 57
Anti-IFN-γ	+	382 ± 53
Anti-IL-10	+	470 ± 64
C57BL/6		
None	-	90 ± 3
Anti-TNF-α	-	74 ± 2
Anti-IFN-γ	-	68 ± 6
Anti-IL-10	-	76 ± 4
None	+	219 ± 10
Anti-TNF-α	+	328 ± 51
Anti-IFN-γ	+	148 ± 23
Anti-IL-10	+	153 ± 17

Groups of 10 BALB/c or C57BL/6 mice were treated with PBS or mAb to TNF-α, IFN- γ or IL-10 on days -1, 1, 3, 7 and 10 p.i. Each dose was 0.5 mg of antibody in ascites diluted in PBS and given i.p. Half the mice from each strain were infected with 10^4 pfu R-MuLV i.p. on day 0 while uninfected mice received PBS. After 2 weeks, the spleen was taken from each mouse and weighed in tared tubes. Data expressed as mean spleen weight of 5 mice ± SEM.

Table 3.6 In vivo *neutralisation of TNF-α and IFN-γ during R-MuLV infection of C57BL/6 mice*

Cytokine neutralising antibody	R-MuLV	Spleen weight
PBS	-	78 ± 3
TNF-α	-	74 ± 11
IFN-γ	-	75 ± 5
TNF-α + IFN-γ	-	68 ± 2
PBS	+	198 ± 28
TNF-α	+	127 ± 7
IFN-γ	+	172 ± 21
TNF-α + IFN-γ	+	139 ± 11

Groups of 10 C57BL/6 mice were treated with PBS or mAb to TNF-α, IFN-γ or both TNF-α and IFN-γ on days -1, 1, 3, 7 and 10 p.i. Each dose was 0.5 mg of antibody in ascites diluted in PBS and given i.p. in a 200 µl volume. Half the mice from each group were infected with 10^4 pfu R-MuLV i.p. on day 0 while uninfected mice received inert carrier alone. After 2 weeks, the spleen was taken from each mouse and weighed in tared tubes. Data expressed as mean spleen weight of 5 mice ± SEM.

Table 3.7 *Growth of R-MuLV in IFN-γ $R^{+/+}$ or IFN-γ $R^{o/o}$ mice*

Mouse Strain	Weeks Post Infection	Plasma Antigen[a]	Spleen Weight (mg)	Mean Spleen Weight[b]
IFN-γ $R^{+/+}$	Uninfected	32, 32, 32, 32	169, 144, 82, 80	118 ± 22
	1	512, 256, 128, 128	175, 149, 133, 131	145 ± 11
	2	128, 64, 32, 32	131, 119, 118, 117	121 ± 3
	3	512, 256, 256, 32	414, 365, 243, 78	275 ± 75
IFN-γ $R^{o/o}$	Uninfected	32, 32, 32, 32	55, 54, 51, 46	51 ± 2
	1	512, 256, 256, 256	264, 234, 231, 146	219 ± 25[d]
	2	64, 64, 32[c]	141, 131, 109	127 ± 10[d]
	3	128, 128, 64, 64	123, 119, 109, 96	112 ± 6[d]

Groups of 4 IFN-γ $R^{+/+}$ or IFN-γ $R^{o/o}$ mice were given 10^4 pfu R-MuLV i.p. 1, 2 or 3 weeks previously. Blood was collected from each mouse by tail vein puncture. Plasma was separated from blood cells and was stored at -20°C until assayed for viral antigen. The spleen was taken from each mouse and weighed in tared, sterile tubes.

a) Endpoint of R-MuLV ELISA of R-MuLV antigen in plasma

b) Mean spleen weight of 4 mice ± SEM

c) One mouse died 13 days p.i. from unknown causes

d) Significantly different ($p<0.001$) when compared to spleen weight of uninfected mice

increase in spleen weight in 3 out of 4 mice, however due to range of the data this difference is not statistically significant (p=0.09, Welch's *t* test).

Levels of viral antigen in the plasma of IFN-γ $R^{+/+}$ mice was biphasic. High levels plasma viral antigen were found at 1 and 3 weeks p.i. while somewhat lower levels were found at week 2 (Table 3.7). The patterns of plasma viral antigen levels in IFN-γ $R^{o/o}$ mice were similar to those found in IFN-γ $R^{+/+}$ mice at 1 and 2 weeks. In contrast, levels of viral antigen titres in plasma at 3 weeks p.i. were lower in IFN-γ $R^{o/o}$ mice compared with IFN-γ $R^{+/+}$ mice.

3.3.4.2 Histological analysis of spleen from IFN-γ $R^{o/o}$ and IFN-γ $R^{+/+}$ mice following R-MuLV infection

R-MuLV infection had significant effects on the cellular composition of the spleen of IFN-γ $R^{o/o}$ and IFN-γ $R^{+/+}$ mice. Early in infection of IFN-γ $R^{+/+}$ mice, small pockets of progenitor haematopoietic cells appeared in the red pulp, however spleen architecture was largely unaltered (Figure 3.4). At 3 weeks p.i. the areas of progenitor haematopoietic cells in the red pulp were extensive and there was significant disruption of splenic architecture. In contrast, there were large areas of progenitor haematopoietic cells in the spleen and moderate disruption of normal spleen morphology at 1 week p.i. in IFN-γ $R^{o/o}$ mice (Figure 3.5). The area of progenitor haematopoietic cells in the red pulp was reduced at 2 weeks p.i. and at 3 weeks these progenitor haematopoietic cells were largely absent. The identity of the haematopoietic cells mentioned above is unclear (Dr A. Hapel, Division of Clinical Sciences, JCSMR, personal communication), however it seems likely that they are progenitor erythroid cells (Rauscher, 1962; Hanna *et al.*, 1970a).

3.3.4.3 Effect of R-MuLV infection on spleen cell proliferation of IFN-γ $R^{o/o}$ and IFN-γ $R^{+/+}$ mice

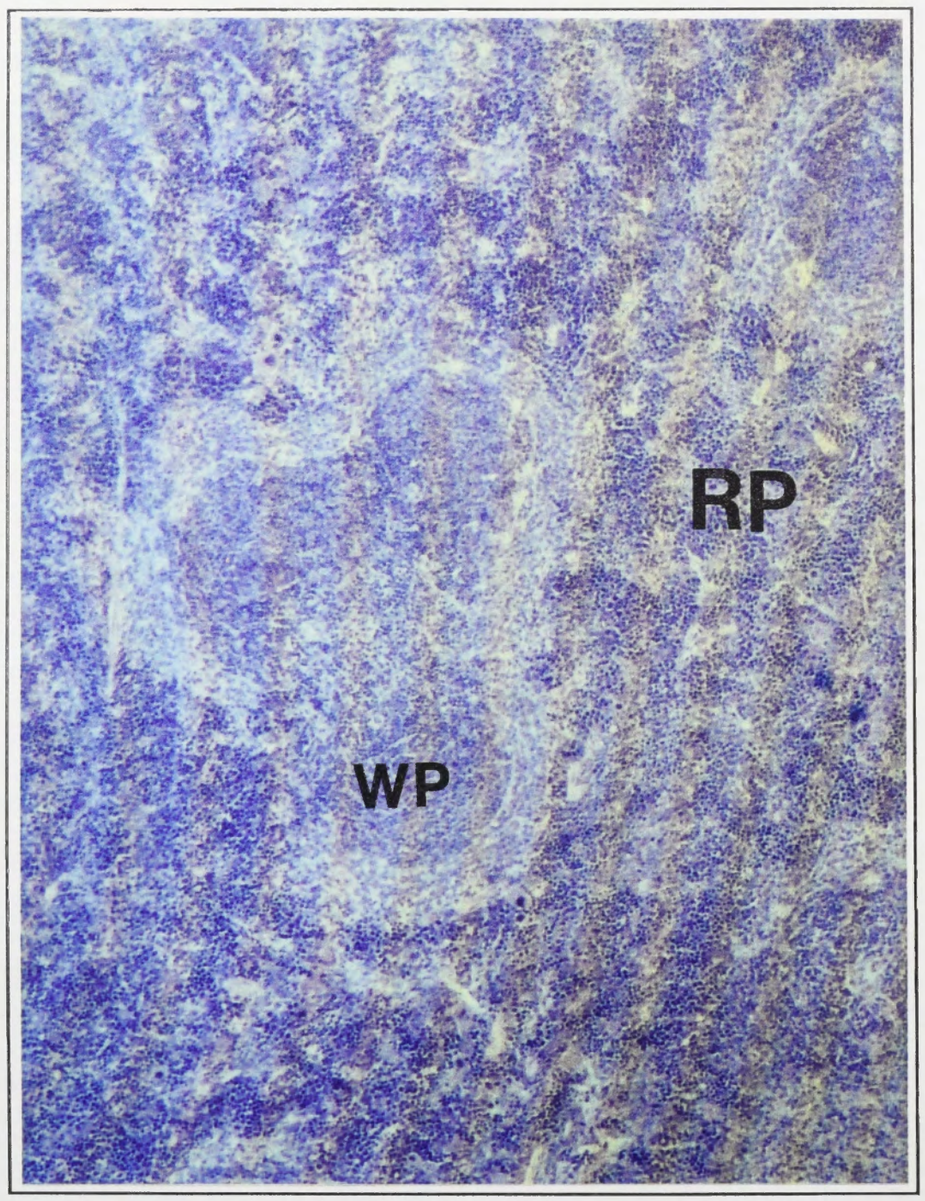
A feature of R-MuLV infection is the marked suppression of spleen lymphocyte proliferation. It was therefore of interest to determine if R-MuLV induced immunosuppression was dependent upon IFN-γ activity. The ability of spleen cells from IFN-γ $R^{o/o}$ and IFN-γ $R^{+/+}$ mice

Figure 3.4 ***Histology of spleens from R-MuLV infected IFN-γ R$^{+/+}$ mice***

Spleens were taken from groups of 4 uninfected IFN-γ R$^{+/+}$ mice or mice which had been infected for 1, 2 or 3 weeks with R-MuLV. The spleens were collected and fixed in neutral buffered formaldehyde. Sections were prepared and stained with May Grünwald Giemsa. Sections shown are representative of 4 spleens. Magnification x40 is shown. White pulp (WP), Red pulp (RP) and Haematopoietic progenitor cells (→) are indicated.

Normal spleen morphology is illustrated in micrograph of spleen from uninfected mice. White and red pulp are well delineated. At 1 and 2 weeks post infection pockets of progenitor haematopoietic cells were found in the red pulp. At 3 weeks p.i. progenitor haematopoietic cells had infiltrated large areas of red pulp. The photomicrograph of spleen at 3 weeks shows white pulp surrounded by red pulp largely comprised of progenitor haematopoietic cells.

Uninfected



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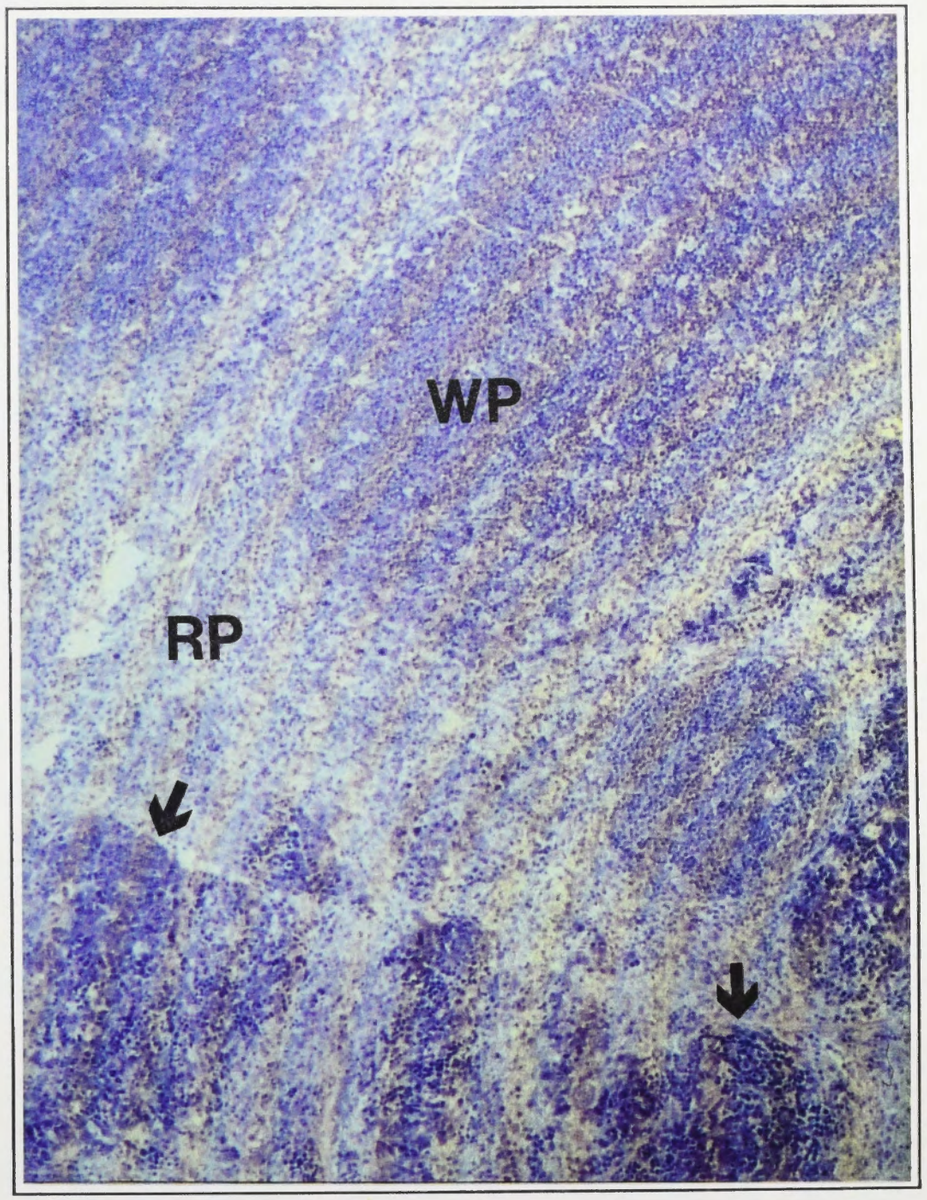

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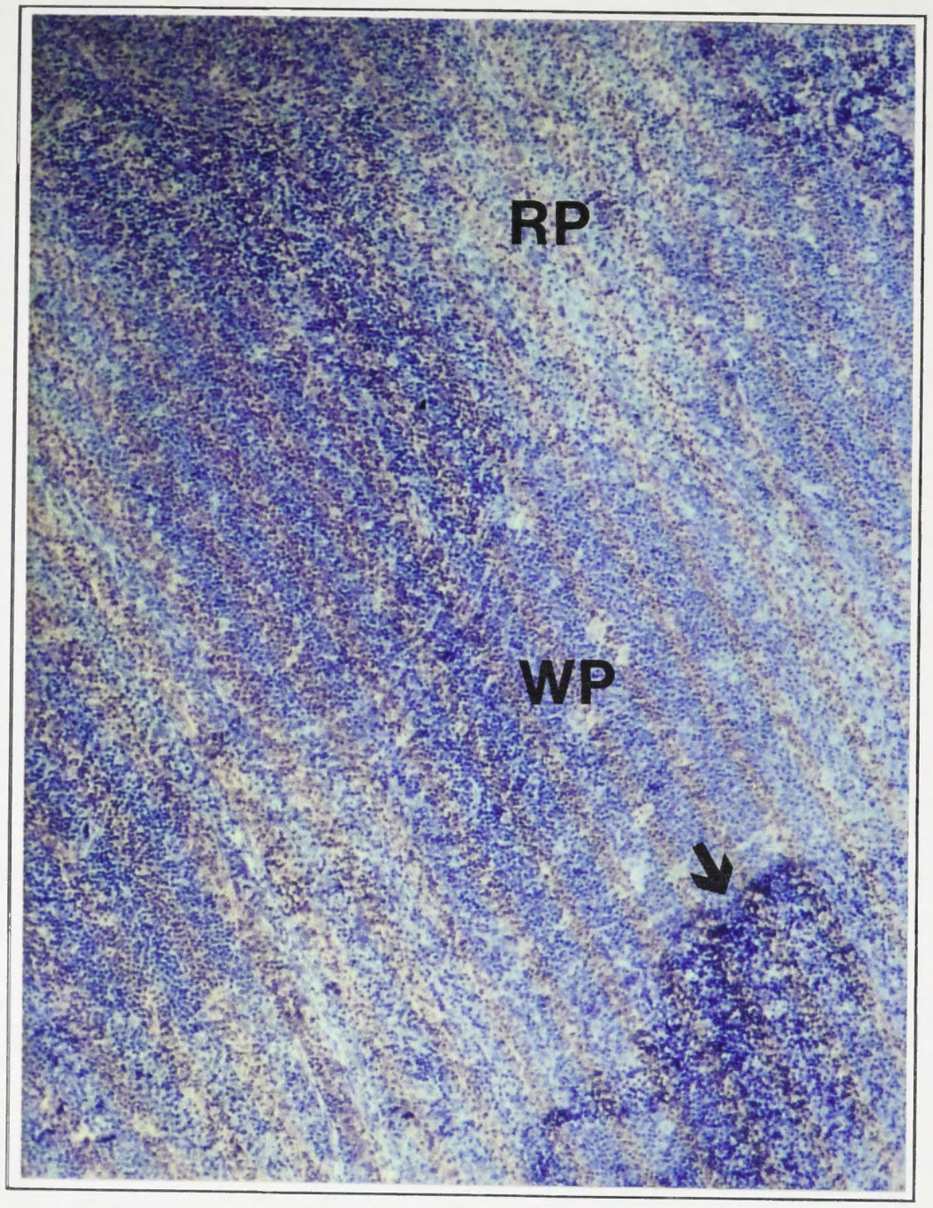


3

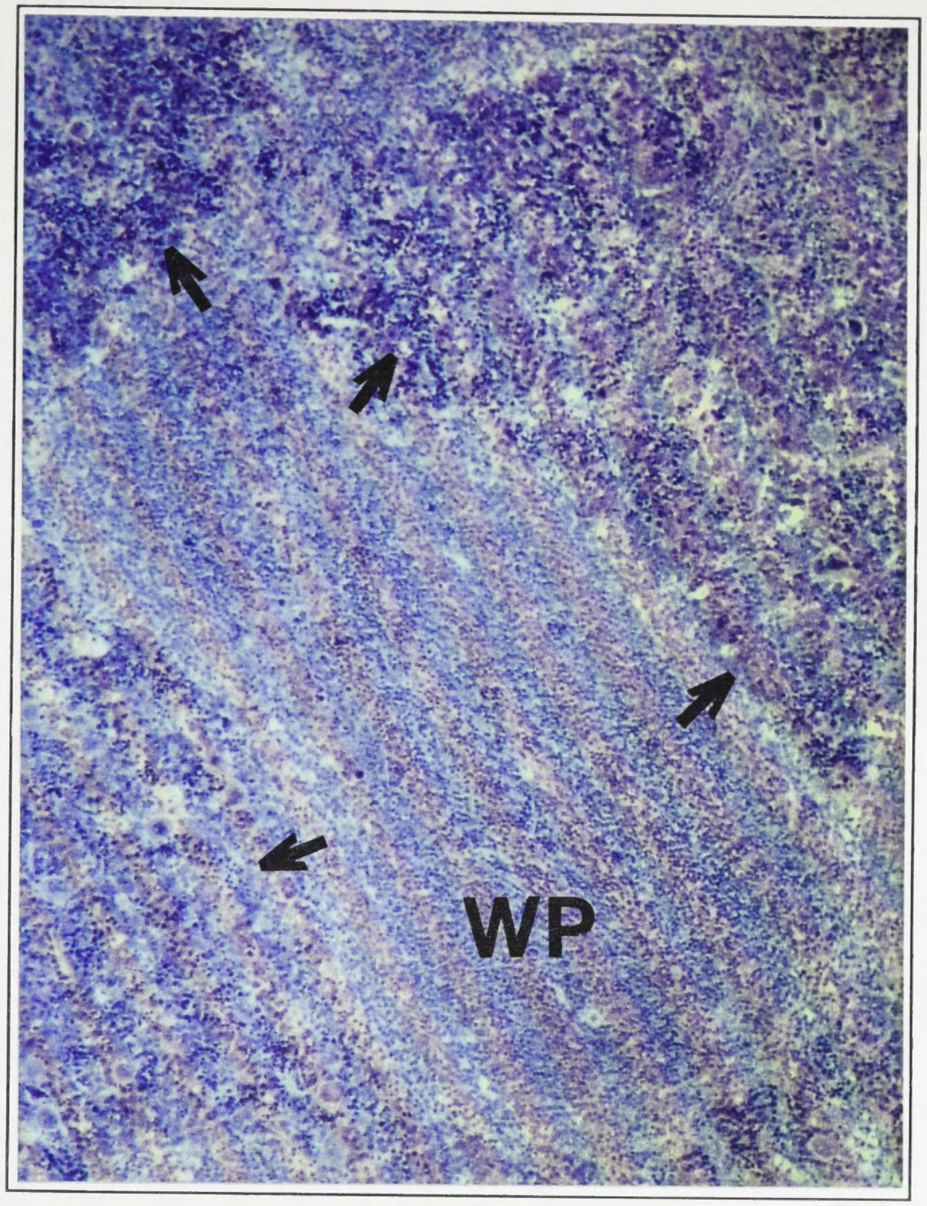
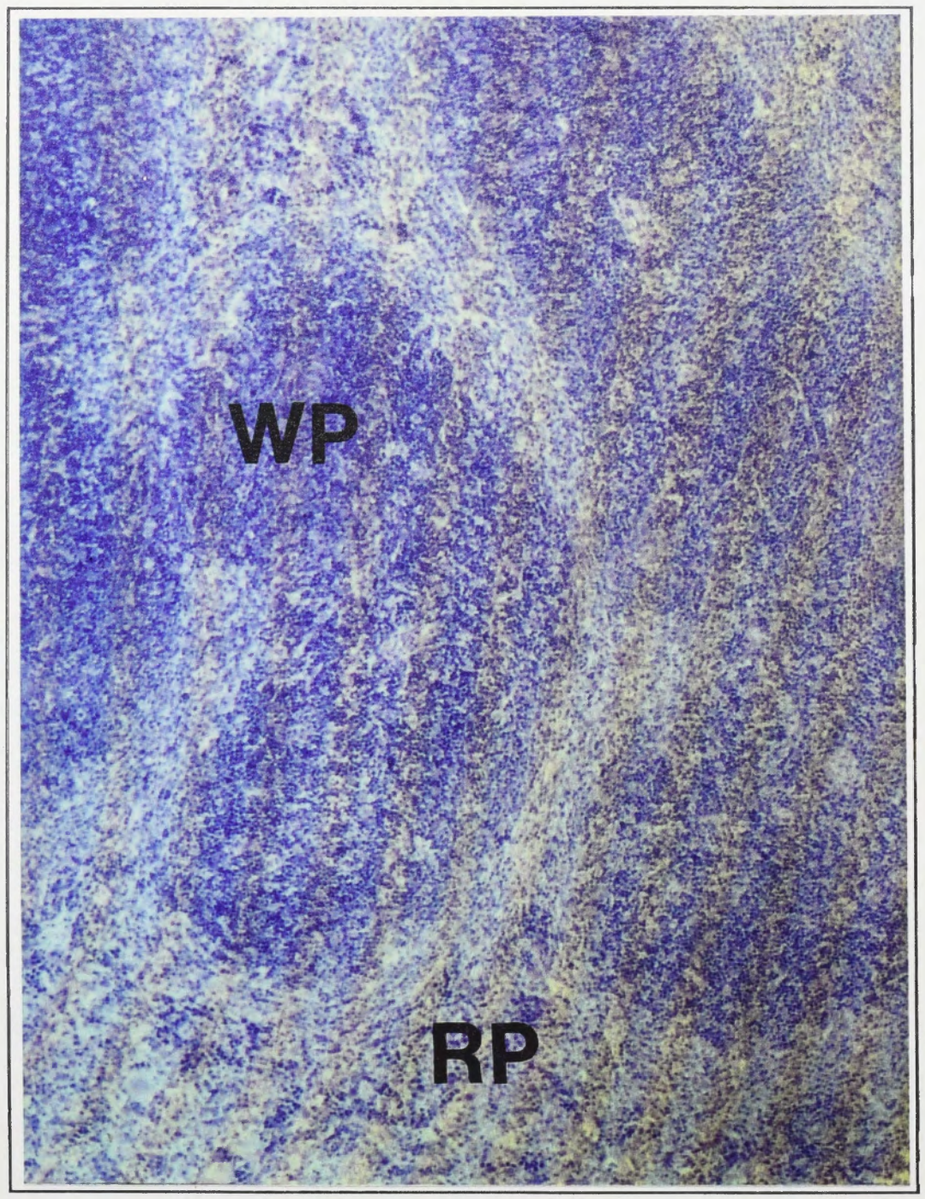


Figure 3.5 ***Histology of spleens from R-MuLV infected IFN-γ R$^{o/o}$ mice***

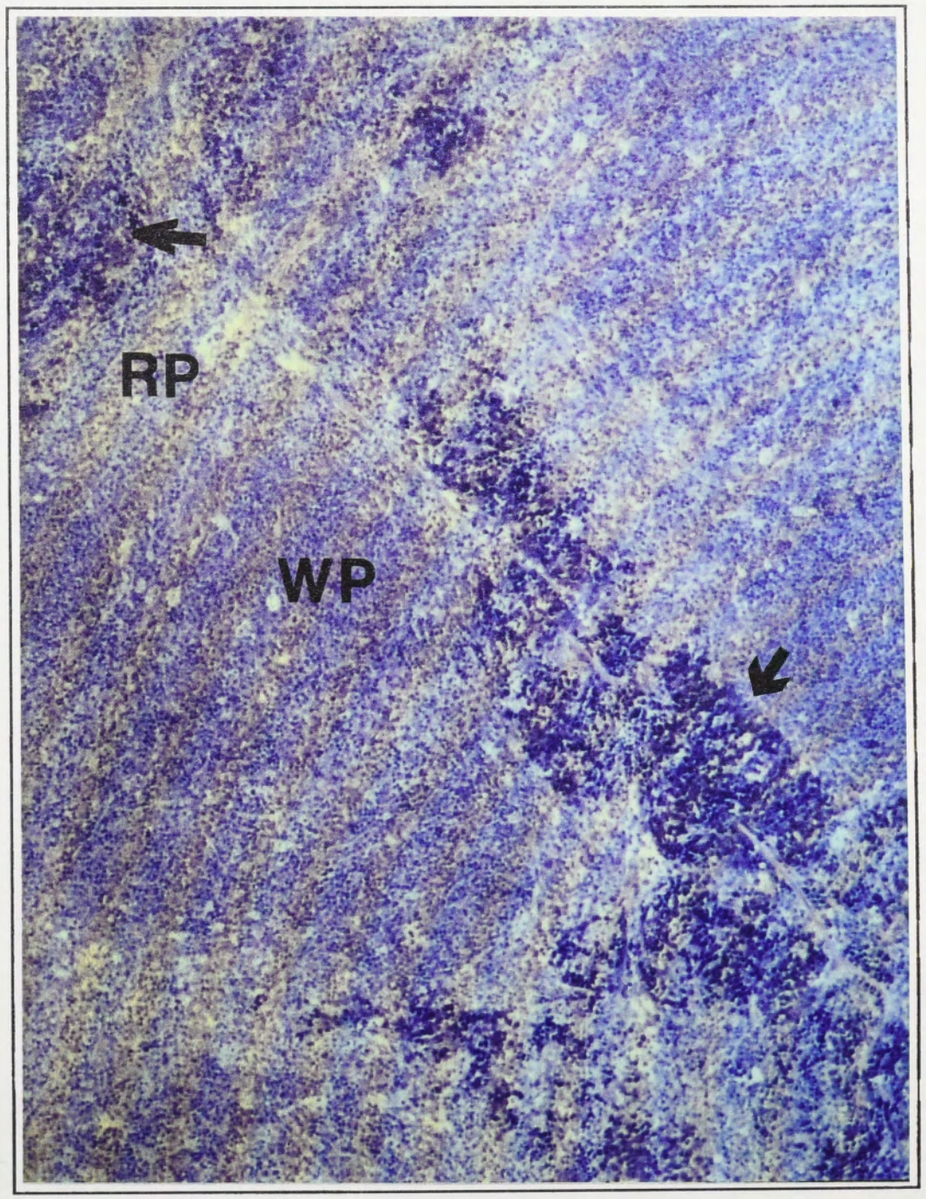
Spleens were taken from groups of 4 uninfected IFN-γ R$^{o/o}$ mice or mice which had been infected for 1, 2 or 3 weeks with R-MuLV. The spleens were fixed for histology in neutral buffered formaldehyde. Sections were prepared and stained with May Grünwald Giemsa. Sections shown are representative of 4 spleens. Magnification x40 is are shown for all sections. White pulp (WP), Red pulp (RP) and Haematopoietic progenitor cells (→) are indicated.

The micrograph of spleen from uninfected mice shows normal spleen morphology. At 1 week p.i. progenitor haematopoietic cells were widespread throughout the red pulp. At 2 weeks p.i. there were only several pockets of progenitor haematopoietic cells in the red pulp, while at 3 weeks these cells were largely absent from the spleen.

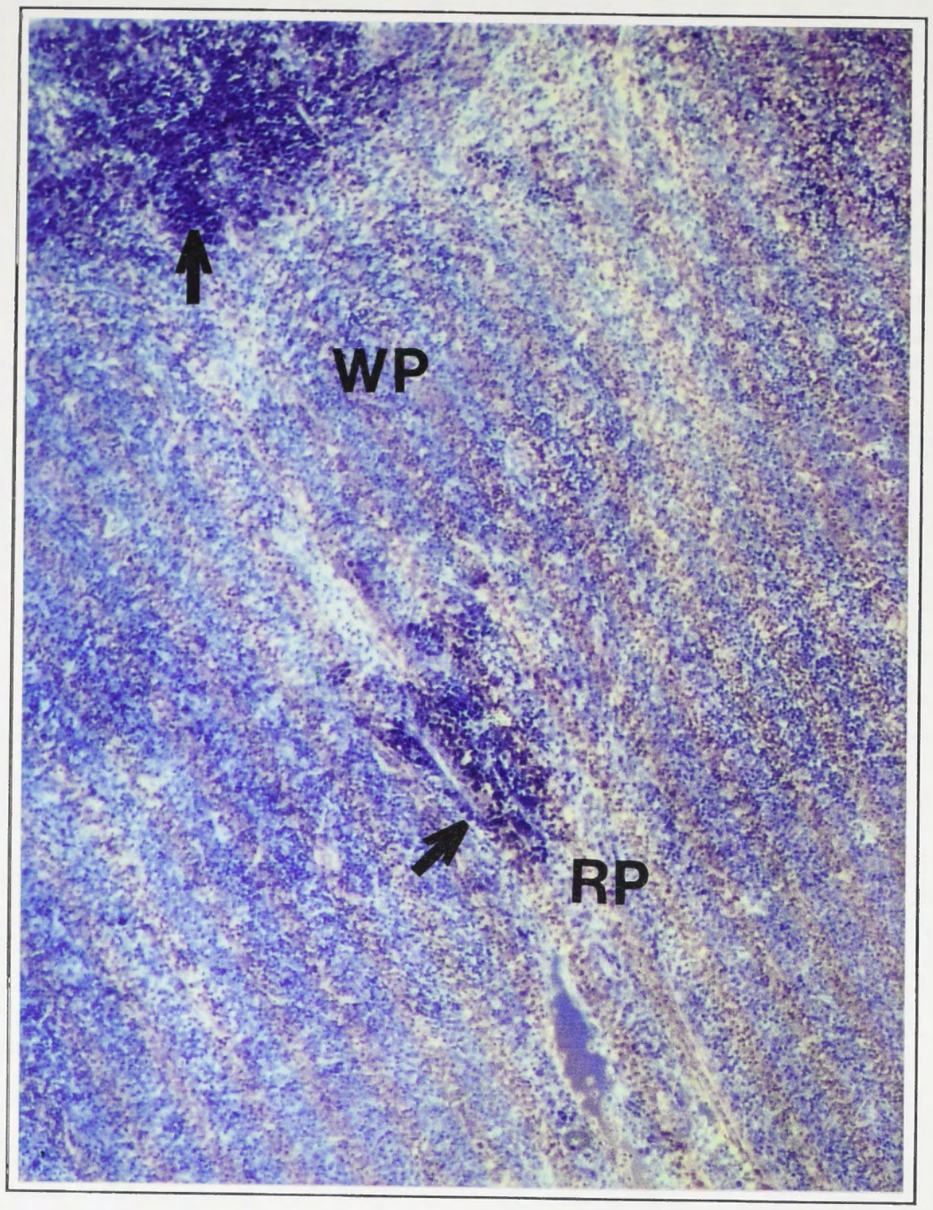
Uninfected



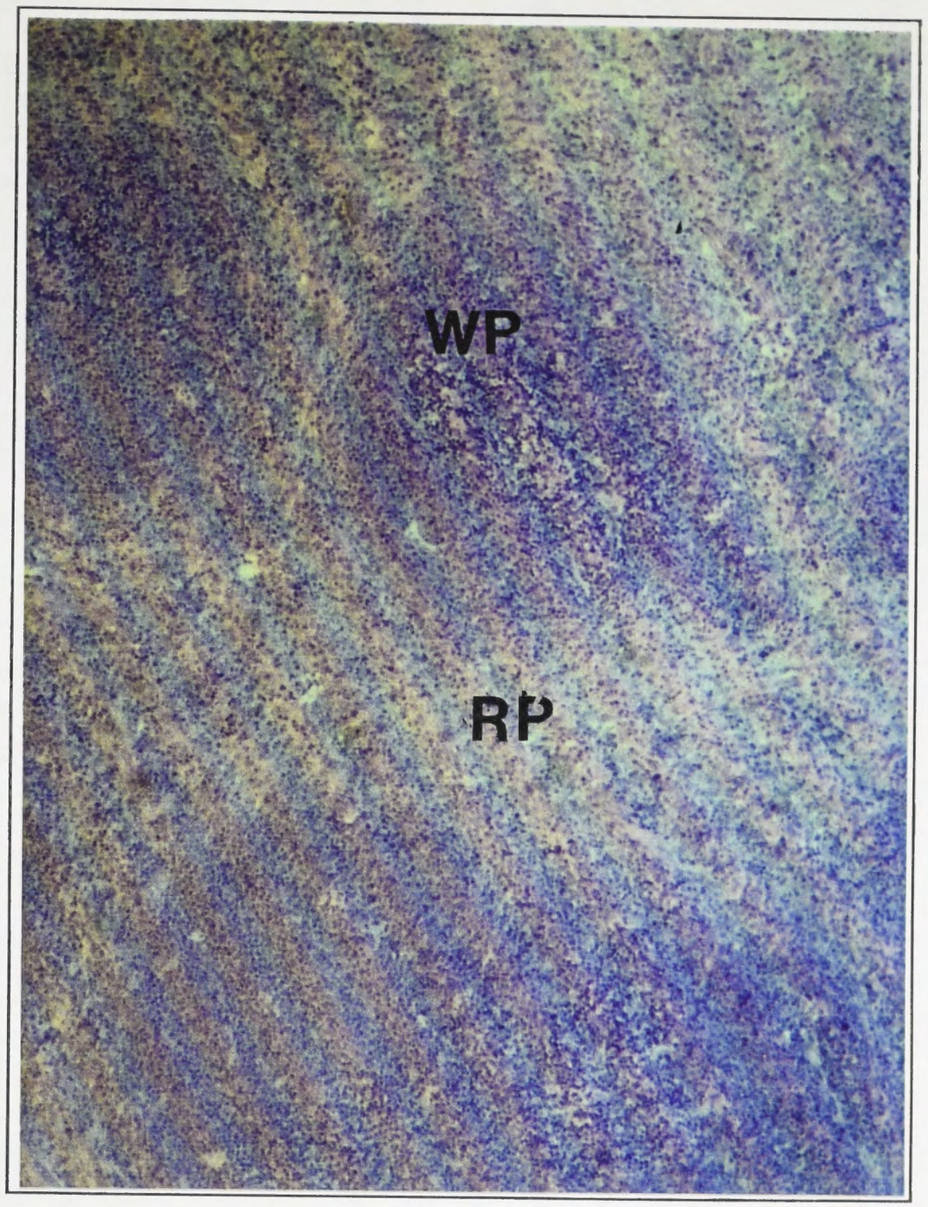
1



2



3



to proliferate in response to stimulation with anti-CD3 or LPS was investigated.

The proliferation of anti-CD3 stimulated spleen cells from IFN-γ $R^{+/+}$ mice was unaltered at 7 and 14 days p.i. but was markedly suppressed at day 21. The proliferative response of spleen cells from wild type mice to LPS stimulation was also rapidly suppressed following R-MuLV infection. The suppression persisted and was greatest at 21 days p.i. In sharp contrast, the response of spleen cells from IFN-γ $R^{o/o}$ mice was largely unaltered at all times p.i. examined (Figure 3.6). In addition, LPS induced proliferation by spleen cells from IFN-γ $R^{o/o}$ mice was also unaffected by R-MuLV infection (Figure 3.6). These data suggest that suppressed lymphocyte proliferation associated with R-MuLV infection may be dependent upon IFN-γ activity.

3.3.5 Effect of *in vivo* depletion of $CD4^+$ or $CD8^+$ cells on R-MuLV induced splenomegaly

In adoptive transfer studies it was demonstrated that both $CD4^+$ and $CD8^+$ spleen cells from R-MuLV immunised mice were required to confer maximal protection against R-MuLV to naive BALB/c mice (Hom *et al.*, 1991). To extend this further, the role of cells expressing CD4 or CD8 in mediating resistance or susceptibility to R-MuLV infection was investigated by depleting mice of these cell subsets during virus challenge. Spleen weight on day 21 was used as a measure of virus growth and disease progression (Chirigos, 1964).

The efficiency with which T cell subsets were depleted was determined by flow microfluorometry of lymph node cells stained for CD4 or CD8 expression. The depletion protocol routinely eliminated greater than 90% of cells expressing CD4 or CD8 (data not shown). The proportion of cells expressing CD4 or CD8 in lymph nodes of mice given ascites from the rat IgG2b control were similar to those of mice receiving PBS alone (data not shown).

Treatment with anti-CD4 antibody decreased R-MuLV induced splenomegaly ($p<0.015$) in infected C57BL/6 mice (Table 3.8). In contrast,

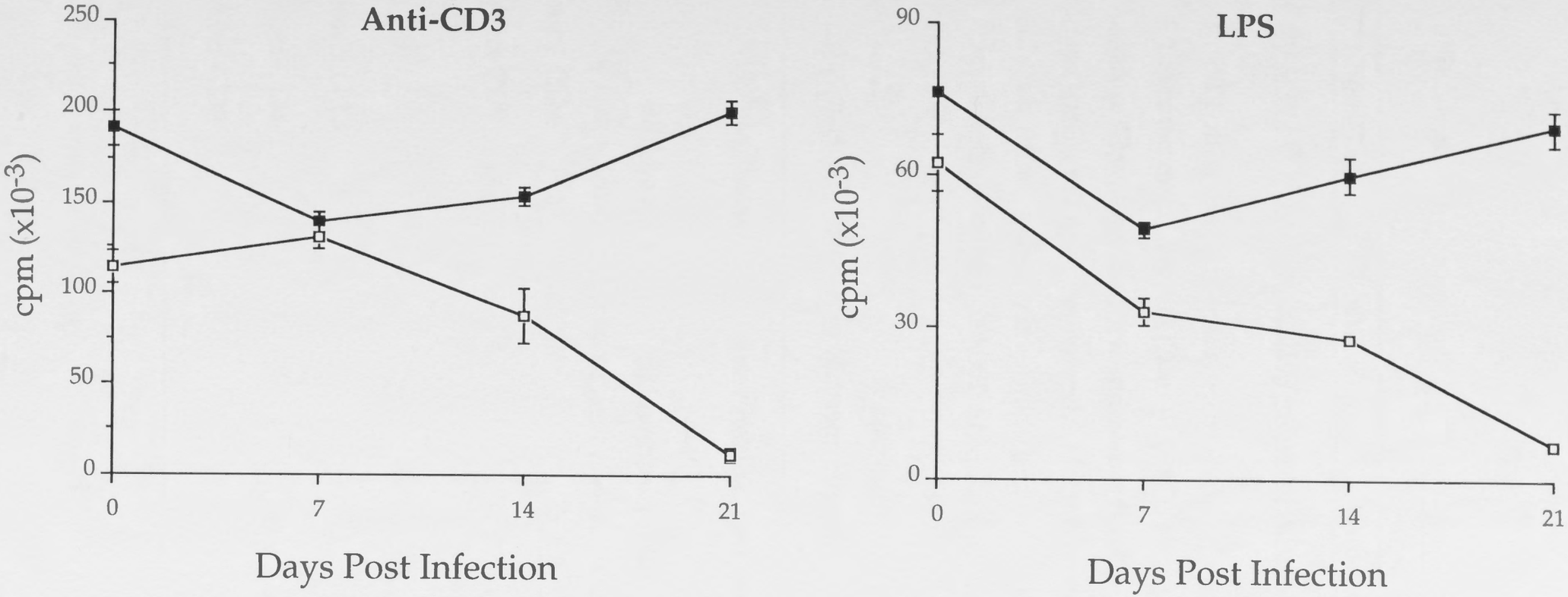


Figure 3.6 ***Proliferation of spleen cells from IFN-γ R$^{o/o}$ IFN-γ R$^{+/+}$ following R-MuLV infection***

Spleens were removed from IFN-γ R$^{o/o}$ (–■–) and IFN-γ R$^{+/+}$ (–□–) which were uninfected or infected with 10^4 pfu R-MuLV 7, 14 or 21 days. The spleens taken at each time point were pooled and single cell suspensions were prepared as described in section 2.2.8. Spleen cells were cultured with immobilised anti-CD3 antibodies or LPS for 72 h and labelled with 0.5 µCi [^{3}H]-TdR for a further 16 h. Data points indicate the mean proliferation of 4 replicate culture (± SEM) as measured by [^{3}H]-TdR incorporation. Data shown is from a single experiment.

Table 3.8 ***R-MuLV disease in mice depleted of cells expressing CD4 or CD8***

Groups of 8 or 10 BALB/c or C57BL/6 mice were treated with PBS or ascites containing mAb against CD4 (GK 1.5) or CD8 (D9). In experiment 2 ascites containing rat IgG 2b antibody reactive with an irrelevant antigen (SFR8-B6) was used as an ascites control. Mice were given 0.5 mg of each antibody on days -1, 1, 3, 7 and 10 and then every 4 days until day 21. Ascites was diluted in PBS and given i.p. in 200 µl. Half the mice in each group were given 10^4 pfu/ml i.p. on day 0. Spleen weight at day 21 p.i. was used as an estimate of disease progression.

a) Mean spleen weight of 5 mice (experiment 1) or 4 mice (experiment 2) ± SEM

b) Significantly greater ($p<0.05$) than spleen weight of infected mice given PBS

c) Significantly less ($p<0.001$) than spleen weight of mice given PBS

d) Significantly less ($p<0.05$) than spleen weight of infected mice given PBS

Table 3.8 (cont)

Antibody	R-MuLV	Mean Spleen weight (mg)[a] Experiment 1	Experiment 2
BALB/c			
PBS	-	95 ± 3	134 ± 7
Rat IgG 2b	-	ND	159 ± 17
Anti-CD4	-	102 ± 4	131 ± 14
Anti-CD8	-	95 ± 4	132 ± 11
PBS	+	843 ± 97	983 ± 30
Rat IgG 2b	+	ND	1052 ± 68
Anti-CD4	+	467 ± 175	541 ± 32[c]
Anti-CD8	+	1202 ± 74[b]	948 ± 53
C57BL/6			
PBS	-	78 ± 5	97.5 ± 11
Rat IgG 2b	-	ND	102 ± 7
Anti-CD4	-	75 ± 3	88 ± 13
Anti-CD8	-	74 ± 5	82 ± 4
PBS	+	174 ± 11	223 ± 35
Rat IgG 2b	+	ND	219 ± 17
Anti-CD4	+	121 ± 11[d]	109 ± 9[d]
Anti-CD8	+	295 ± 24[b]	310 ± 23[b]

depletion of $CD8^+$ cells during R-MuLV infection led to increased spleen weight ($p<0.05$). These data indicate that $CD4^+$ and $CD8^+$ cells either promote or limit R-MuLV induced splenomegaly, respectively, in C57BL/6 mice.

Splenomegaly in BALB/c mice depleted of $CD4^+$ cells compared with mice receiving PBS alone was reduced in both experiments (Table 3.8). However, due to the range of the data, the difference was statistically significant only in experiment 2 ($p<0.001$). These results suggest that $CD4^+$ cells may be required for optimal development of disease associated with R-MuLV. Virus induced splenomegaly was elevated in BALB/c mice which were depleted of $CD8^+$ cells ($p=0.02$, experiment 1), however in experiment 2 there was no difference in spleen weight compared to R-MuLV infected PBS treated mice ($p>0.1$). This variability in spleen weight limits the conclusions which may be drawn about the role of $CD8^+$ cells in R-MuLV pathogenesis in BALB/c mice.

3.4 DISCUSSION

The type of immune responses generated toward an infectious agent often correlate with resistance or susceptibility to infection. Cytokines play an integral role in determining the class of the immune response which is induced (Ramshaw *et al.*, 1992; Powrie and Coffman, 1993a). Thus, the cytokines produced following infection of C57BL/6 or BALB/c mice may be expected to influence the type of antiviral immune response generated toward R-MuLV and therefore the resistance or susceptibility of each strain of mice. The experiments in this chapter were designed to determine the patterns of cytokine expression as well as the role of cytokines in resistance or susceptibility to R-MuLV induced disease. As T cell subsets regulate immune responses via the cytokines they produce (Mosmann and Coffman, 1989; Seder and Paul, 1994) the role of these cells in resistance or susceptibility to R-MuLV infection was also examined.

Expression of IFN-γ mRNA was slightly elevated in the spleens of infected BALB/c and C57BL/6 mice. Despite this increase, spleen cells from R-MuLV infected C57BL/6 mice and BALB/c mice with established disease produced decreased levels of IFN-γ following anti-CD3 stimulation. These observations indicate that *in vitro* production of IFN-γ does not directly correlate with the expression of IFN-γ mRNA *in vivo*. One interpretation of this result is that IFN-γ may be produced *in vivo* by cells that do not respond to anti-CD3 stimulation, such as NK cells. It is therefore notable that IFN-γ mRNA expression by splenocytes was induced following LP-BM5 MuLV infection of C57BL/6 mice, while Con A stimulated IFN-γ protein synthesis was progressively reduced (Pitha *et al.*, 1988; Gazzinelli *et al.*, 1992; Uehara *et al.*, 1994). Recent studies have suggested that NK cells may be the source of elevated IFN-γ expression in LP-BM5 infected mice as LPS induced IFN-γ production by NK cells was increased in LP-BM5 infected mice (Uehara *et al.*, 1994; Morse *et al.*, 1995). Interestingly, IFN-γ mRNA expression by splenocytes was also elevated during murine cytomegalovirus (MCMV) infection, while Con A stimulated IFN-γ protein synthesis was progressively

reduced. In addition, LPS stimulated IFN-γ production by splenocytes was increased following MCMV infection (G. Karupiah, JCSMR personal communication). Similar experiments using spleen cells from R-MuLV infected mice may clarify the contribution of NK cells to the levels of IFN-γ mRNA which were detected *in vivo.*

Post transcriptional regulation of IFN-γ expression may also explain the disagreement between IFN-γ mRNA levels and IFN-γ production *in vitro.* Regulation of IFN-γ expression is thought to occur primarily at the levels of transcription (Farrar and Schrieber, 1993), however it is notable that IFN-γ mRNA is found at high levels in draining lymph nodes of mice infected with ectromelia while IFN-γ protein was not detected (G. Karupiah, JCSMR personal communication). It is not known at this time how this effect is mediated.

In vitro IL-2 production by spleen cells from BALB/c mice at 1 and 2 weeks p.i. was similar to that of uninfected controls. IL-2 production by spleen cells from infected C57BL/6 mice was also largely unaltered following R-MuLV infection. However, IL-2 production by BALB/c mice at 3 weeks p.i. was markedly suppressed which may lead to reduced T cell proliferation at this time (Figure 2.1). Interpretation of this data is complicated by the fact that IL-2 is a growth factor for many cells (Swain, 1991) and therefore the lower levels found may reflect increased adsorption of IL-2. This possibility may be examined by determining IL-2 receptor expression by spleen cells or assessing the ability of these cells to bind IL-2.

As IL-2 is central to the activation and proliferation of T cells (Swain, 1991) decreased IL-2 production may be integral to the suppressed response of spleen T cells found in R-MuLV infected mice (see Figure 2.1). However, *in vitro* IL-2 production by spleen cells from infected BALB/c mice was not markedly suppressed until 3 weeks p.i. while T cell proliferation was significantly impaired at 1 and 2 weeks p.i. Furthermore, spleen cells from infected C57BL/6 mice produced IL-2 at levels similar to those produced by spleen cells from uninfected mice while proliferation of T cells from infected mice was reduced (Figure 2.1).

These data suggest that suppressed T cell proliferation may not be due to insufficient IL-2 production. Indeed, lower levels of IL-2 may reflect impaired responses by T cells following ligation of the T cell receptor or T cells receiving weak costimulatory signals.

IL-2 mRNA expression by spleen cells from infected BALB/c and C57BL/6 mice was suppressed at 3 weeks p.i. IL-2 production by anti-CD3 stimulated spleen cells from R-MuLV infected BALB/c mice was also reduced at 3 weeks p.i. In contrast, *in vitro* IL-2 production by spleen cells from C57BL/6 mice was largely unaltered at 3 weeks p.i. The reason for this difference is unclear, however as IL-2 transcription occurs following the stimulation of T cell it may be that T cell activation is reduced in R-MuLV infected C57BL/6 mice. That T cell stimulation *in vitro* is provided by anti-CD3 antibodies raises the possibility that reduced IL-2 transcription *in vivo* may be due to defective antigen presentation in these mice. It may therefore be interesting to assess the ability of cells such as macrophages and dendritic cells from C57BL/6 mice to present antigen following R-MuLV infection.

IL-6 production by spleen cells from C57BL/6 mice was elevated following R-MuLV infection. IL-6 expression has been shown to be induced during infection with a number of viruses, including vaccinia virus (Carpenter *et al.*, 1994), Sendai virus (Sehgal *et al.*, 1988) and HIV (Nakajima *et al.*, 1989). As IL-6 has been shown to activate T cells, promote CTL generation and augment antibody production by B cells (van Snick, 1990) the increased IL-6 production in resistant mice is consistent with a role of this factor in the generation of antiviral immunity in these animals. Peak IL-6 production by spleen cells from uninfected and infected BALB/c or C57BL/6 mice was found to be dependent on adherent cells (Table 3.2). The exact identity of the adherent cells was not determined, however a majority did not express Thy 1.2 which indicates that they were not T cells. These data do not demonstrate which cells produce IL-6, however it is notable that plastic adherent cells in human peripheral blood or spleens of vaccinia infected mice are the major source of IL-6 produced following *in vitro* stimulation (Aarden *et al.*, 1987; Carpenter *et al.*, 1994).

The pattern of *in vitro* IL-2 and IFN-γ production by spleen cells from R-MuLV-infected BALB/c mice (Table 3.1) is similar to that produced by phorbol ester and calcium ionophore stimulated spleen cells from F-MuLV infected BALB/c mice (Soldaini *et al.*, 1991). In contrast, the capacity of splenocytes from F-MuLV infected BALB/c mice to produce IL-2 and IFN-γ following Con A stimulation was suppressed as early as 1 week p.i. and completely inhibited by week 3 (Lopez-Cepero *et al.*, 1988; Matteucci *et al.*, 1989; Shen *et al.*, 1991; Soldaini *et al.*, 1991). In addition, Con A stimulated IL-6 production by spleen cells from F-MuLV infected BALB/c mice was markedly suppressed (Faxvaag *et al.*, 1993) while data presented in this thesis showed that R-MuLV infection had little effect on IL-6 production by anti-CD3 stimulated spleen cells from BALB/c mice. These cytokine expression data may reflect differing effects of R-MuLV and F-MuLV on the signalling pathways induced by each stimulus.

IL-4 was not detected in supernatants from anti-CD3 stimulated spleen or lymph node cells from infected mice, however stimulated spleen cells from uninfected BALB/c mice produced low levels of IL-4. These data suggest that spleen and lymph node cells are not primed to produce high levels of IL-4 following R-MuLV infection. These data however, do not exclude the possibility that IL-4 may be produced at low levels nor the possibility that IL-4 may have been consumed by spleen cells during culture. An alternative strategy for determining the frequency of IL-4 secreting T cells by limiting dilution analysis or IL-4 specific ELISPOT assay may further clarify IL-4 expression during R-MuLV infection.

TNF-α was not detected in supernatants from cultured spleen or lymph node cells from uninfected or infected mice. These data suggest that R-MuLV infection does not prime spleen and lymph node cells to secrete high levels of TNF-α following T cell restimulation *in vitro*. This finding is surprising given that T cells secrete TNF-α following *in vitro* stimulation (Beutler and Cerami, 1989). Furthermore, macrophages have been shown to produce TNF-α following exposure to IFN-γ and/or interaction with activated T cells (Stout, 1993) both of which are present in anti-CD3 stimulated cultures. It is possible that membrane bound TNF-α represented a significant component of TNF-α production.

Further experiments which examine the level of membrane bound TNF-α or the TNF-α production by immune cells in response to other stimuli known to induce TNF-α synthesis eg. LPS (Beutler and Cerami, 1989) may examine the effect of R-MuLV infection upon TNF-α synthesis.

In contrast to spleen cells anti-CD3 stimulated lymph node cells from R-MuLV infected C57BL/6 or BALB/c mice were found to produce elevated levels of IFN-γ and IL-6 compared to uninfected controls. The dissimilarity between the patterns of cytokine production in spleens and lymph nodes may be explained by the differing cellular composition of these organs. Moreover, a number of studies have demonstrated that lymphocytes from different sites produce different cytokines. Hoiden and Moller, (1991) reported that Con A stimulated IFN-γ production by spleen cells was greater than that produced by lymph nodes. Plastic adherent cells were found to regulate cytokine secretion by spleen or lymph node cells (Hoiden and Moller, 1991). Daynes *et al.*, (1990) postulated that macrophages mediate these effects by local activation of steroid hormones which modulate cytokine production by lymphocytes. Virus infection introduces a number of other possible regulatory mechanisms. Retroviral peptides may serve as immunogenic antigens and as immunosuppressive molecules (Snyderman and Ciancolo, 1984). The relative levels of R-MuLV in spleen and lymph node was not assessed, however viral replication in the spleens of F-MuLV infected mice exceeds that in the lymph nodes (Isaak *et al.*, 1979) and therefore R-MuLV levels may play a significant role in influencing the expression of cytokines. As discussed in Chapter 2, identification of immune cell populations which are infected with R-MuLV may shed light on the role of the virus in different responses exhibited by spleen and lymph node cells.

Cytokine production by spleen and lymph node cells from uninfected and R-MuLV infected mice was induced *in vitro* using anti-CD3 antibodies. As anti-CD3 antibodies stimulate T cells polyclonally (Maraskovsky *et al.*, 1991), the cytokines produced reflect the result of stimulating virtually all T cells in the lymph node or spleen cultures. This is in contrast to antigen stimulated cultures where cytokine production follows

stimulation of antigen specific T cells. During an immune response the number of antigen specific T cells which are activated and primed to express cytokines are increased compared with levels found in naive animals (Morris *et al.*, 1992). It may therefore be postulated that changes in cytokine production between polyclonally activated immune cells from naive mice and mice undergoing an immune response reflect the nature of that immune response. Indeed, there are many examples where similar cytokines are produced by immune cells which have been restimulated *in vitro* with either antigen or polyclonal T cell stimuli such as anti-CD3 antibodies or Con A during immune responses to viruses (Niemialtowski and Rouse, 1992; Carpenter *et al.*, 1994) and parasites (Gryzch *et al.*, 1991). Moreover, (Kelso *et al.*, 1994) found that *in vitro* cytokine production by lymph node T cells from mice immunised with keyhole limpet haemacyanin depended upon the *in vivo* history of these cells rather than the conditions of *in vitro* restimulation. It seems likely therefore, that similar patterns of cytokines may be produced by R-MuLV-antigen or anti-CD3 stimulated spleen or lymph node cells from R-MuLV infected mice.

It is notable however, that antigen specific immune responses may influence the cytokines produced by non-antigen specific T cells (Kullberg *et al.*, 1992; Coyle *et al.*, 1995). For example, mice infested with *Schistosoma mansoni* exhibit marked deviation of immune responses to non-*S. mansoni* antigens toward type 2 cytokine production and down regulation of CMI responses (Kullberg *et al.*, 1992; Actor *et al.*, 1994). In this context, it is conceivable that the cytokines produced by non-antigen specific T cells following polyclonal activation *in vitro* may influence cytokines produced by antigen specific T cells. In light of this possibility it is clear that antigen dependent restimulation of immune cells is required to clarify T cell dependent cytokine production during R-MuLV infection.

A wide spectrum of cytokines may be expressed during an immune response. There are a number of examples where the pattern of cytokines expressed are markedly polarised towards either type 1 or type 2 cytokines (Sher *et al.*, 1992; Romagnani, 1994). Examination of cytokine expression following R-MuLV infection indicated that both susceptible and resistant

mice predominantly expressed type 1 cytokines. These observations suggest that the susceptibility exhibited by BALB/c mice is not due to a bias towards type 2 cytokine production which may be inappropriate for responses favouring virus clearance. Similar observations have been made by (Brenner *et al.*, 1994) who showed that HSV-1 resistant and susceptible mice produce type 1 cytokines following infection. Interestingly, in this present study, spleen cells from BALB/c mice consistently produced lower levels of IFN-γ upon restimulation compared with levels produced by splenocytes from C57BL/6 mice. Furthermore, the difference was more pronounced as infection progressed. These observations raise the possibility that resistance to R-MuLV infection exhibited by C57BL/6 mice may be due to expression of high levels of IFN-γ and that BALB/c mice fail to produce sufficient IFN-γ to resist R-MuLV infection. These hypotheses are consistent with the relatively poor anti-R-MuLV immune response generated by BALB/c mice compared with C57BL/6 mice (see Section 1.10).

That certain cytokines are integral to the generation and effector activities associated with an immune response and therefore resistance or susceptibility to infectious agents may be demonstrated using cytokine neutralising antibodies. For example, mice infected with ectromelia (Karupiah *et al.*, 1993a), vaccinia (Ruby and Ramshaw, 1991) or herpes simplex (Smith *et al.*, 1994) viruses exhibit a diminished ability to resolve infection when treated with anti-IFN-γ antibodies. Recent studies with mice lacking functional cytokine or cytokine receptor genes have also demonstrated the role of cytokines in immunity to a wide range of pathogens (Pfeffer *et al.*, 1993; Biron, 1994; Kaufmann, 1994). However, administration of IL-2, IL-4, IL-6, IL-10, IFN-γ or TNF-α neutralising antibodies had little effect on R-MuLV induced disease in either BALB/c or C57BL/6 mice. These observations suggest that, individually, these cytokines may not be essential for the pathogenesis of R-MuLV infection in susceptible BALB/c. These findings also suggest that the resistance to R-MuLV infection exhibited by C57BL/6 mice may not be dependent upon the expression of type 1 cytokines such as IFN-γ or IL-2.

A number of studies have shown that IFN-γ and TNF-α act in concert to augment antiviral activity (Wong and Goeddel, 1986; Wong *et al.*, 1988). Furthermore, IFN-γ and TNF-α are involved in activation of macrophages to effector function (Stout, 1993). Concurrent administration of antibodies to neutralise TNF-α or IFN-γ also failed to alter the resistance of C57BL/6 mice to R-MuLV induced disease. The data presented here suggest that the antiviral or immunoregulatory activities of these factors may not be essential for resistance to R-MuLV in C57BL/6 mice.

Several factors should be considered in the interpretation of experiments described in which cytokine neutralising antibodies. Firstly, the question arises of the use of neutralising antibodies at sufficiently high concentrations at the sites of cytokine synthesis in order to inhibit cytokine activity. The antibodies and protocols used here have been extensively and successfully used elsewhere (Table 3.9) which strongly indicate that inhibitory concentrations of antibody were achieved *in vivo*. For instance, the IFN-γ neutralising antibody, R4-6A2, has been used successfully to inhibit IFN-γ dependent clearance of vaccinia (Karupiah *et al.*, 1990; Ruby and Ramshaw, 1991) and NO induction during vaccinia infection (Rolph *et al.*, 1996). In addition this antibody has been used to inhibit the ability of vaccinia-immune CTL to protect irradiated mice from vaccinia virus infection (Ruby and Ramshaw, 1991). As similar protocols were used in experiments described in this thesis it is difficult to believe that the regime of antibody administration did not achieve sufficient levels of antibody to inhibit cytokine activity during R-MuLV infection. The issue of effective cytokine neutralisation with antibodies may be circumvented by using mice lacking functional cytokines or cytokine receptors and was done here in the case of IFN-γ $R^{o/o}$ mice (see below). A second consideration is that the functions of cytokines often overlap, and therefore the neutralisation of a particular cytokine may not affect the overall response. Thirdly, only a single parameter of virus growth ie. spleen weight was determined. Although relatively simple and widely used (Chirigos, 1964; Ruprecht *et al.*, 1986; Ruprecht *et al.*, 1990); also see Section 2.4), this method may not resolve small but

Table 3.9 *Examples of model systems where cytokine activity has been neutralised by antibodies described in Section 3.3.3*

Cytokine	Clone	*In vivo* Model	References
IFN-γ	R4-6A2	Vaccinia infection	Ruby and Ramshaw, (1991)
		Ectromelia infection	Karupiah *et al.*, (1993)
TNF-α	XT-22	*Schistosoma mansoni* infestation	Gazzinelli *et al.*, (1993)
IL-2	S4B6	Recombinant vaccinia expressing IL-2	Karupiah *et al.*, (1990)
IL-4	11B11	*Leishmania major* infestation,	Chatelain *et al.*, (1992)
		Respiratory syncytial virus vaccination	Tang and Graham, (1994)
IL-6	20F3	*E. coli* infection	Starnes *et al.*, (1990)
IL-10	JES-2A5	Experimental endotoxemia	Marchant *et al.*, (1994)
		Antibody production *in vivo*	Dobber *et al.*, (1994)

possibly significant differences in virus growth. Determination of infectious virus in spleen or plasma as well as viral antigen in these sites may provide a greater insight into the effect of cytokine neutralising antibodies and therefore the role of cytokines in the pathogenesis of R-MuLV infection.

In mice lacking functional IFN-γ receptors, the disease caused by R-MuLV was clearly very different from that observed in control mice. Wild type IFN-γ $R^{+/+}$ mice exhibit little effects of the virus until 3 weeks p.i. where the characteristic splenomegaly involving marked accumulation of progenitor haematopoietic cells in the spleen associated with R-MuLV infection occurred. In contrast, splenomegaly and elevated levels of progenitor haematopoietic cells in the spleen were found early in infection of IFN-γ $R^{o/o}$ mice and were reduced with time p.i. In addition, impaired spleen lymphocyte proliferation associated with R-MuLV infection appeared to be dependent upon IFN-γ activity as the proliferation of cells from IFN-γ $R^{+/+}$ but not IFN-γ $R^{o/o}$ mice was suppressed following R-MuLV infection.

It is difficult to propose a model which might explain the differences in pathogenesis of R-MuLV infection in IFN-γ $R^{+/+}$ and IFN-γ $R^{o/o}$ mice. Both mice exhibited features associated with infection of prototypic susceptible BALB/c mice ie. splenomegaly and accumulation of progenitor haematopoietic cells, however only mice lacking functional IFN-γ receptors seemed to control R-MuLV infection. It therefore appeared that IFN-γ may be required for the susceptibility of mice of the 129/Sv strain to R-MuLV infection. One hypothesis that may be proposed to explain the difference in the R-MuLV induced disease in IFN-γ $R^{+/+}$ and IFN-γ $R^{o/o}$ mice is that IFN-γ acts as a growth factor for major target cells of R-MuLV such as progenitor erythroid cells (Rauscher, 1962; Hanna *et al.*, 1970a; de Both *et al.*, 1978). Alternatively, IFN-γ may activate cells such as macrophages which support progenitor erythroid cell replication via production of the growth factor erythropoietin (Gallacchio and Murphy Jr, 1983; Vogt *et al.*, 1989). However, these hypotheses seem unlikely as increased levels of progenitor haematopoietic cells are present in the spleen of IFN-γ $R^{o/o}$

mice. Furthermore, IFN-γ has been shown to inhibit erythropoiesis in normal (Dybedal *et al.*, 1995; Eng *et al.*, 1995) and F-MuLV infected mice (Means Jr *et al.*, 1994).

It could be postulated that the lack of IFN-γ activity in IFN-γ $R^{o/o}$ mice allows for an alternative and more effective immune response to develop which limits disease. As IFN-γ has been shown to be involved in promoting a CMI response and inhibiting the generation of a humoral immune response (Belosevic *et al.*, 1989; Scharton and Scott, 1993), the IFN-γ $R^{o/o}$ mice may be more resistant to R-MuLV by virtue of generating a humoral immune response. This hypothesis may be tested by determining if R-MuLV infected IFN-γ $R^{o/o}$ mice generate anti-R-MuLV immune responses with the characteristics of a humoral immune responses ie. the expression of high levels of type 2 cytokines and high plasma levels of IgG1 and IgE (Mosmann *et al.*, 1986a; Mosmann and Coffman, 1989). Further experiments may also examine R-MuLV induced disease at later time points to elucidate the outcome of R-MuLV infection in these mice.

Interpretation of data obtained from IFN-γ $R^{o/o}$ mice is complicated by the fact that the mice used here were generated on a 129/Sv background (Huang *et al.*, 1993). These mice are genetically different to mice used elsewhere in this thesis and therefore the validity of comparisons between the data obtained from IFN-γ $R^{o/o}$ mice and the data presented in the rest of this thesis may be limited. Gene knock out mice generated on a BALB/c or C57BL/6 background may therefore be more suitable to answer questions relating to the role of cytokines in the pathogenesis of R-MuLV infection of these mice. Further work is required to resolve the apparent contrast between R-MuLV infection of IFN-γ $R^{o/o}$ mice and BALB/c mice treated with IFN-γ neutralising antibody. It would be interesting to compare the titres of virus and levels of viral antigen in the spleen and plasma in R-MuLV infected BALB/c mice treated with IFN-γ neutralising antibody and IFN-γ $R^{o/o}$ mice.

The data obtained from IFN-γ $R^{o/o}$ mice is also complicated by the fact that these mice have developed in the absence of IFN-γ activity and

therefore may have generated functionally redundant pathways to compensate for this defect. This redundancy is illustrated in mice lacking the T cell growth factor, IL-2. In these mice, the lack of IL-2 is compensated for by other cytokines such as IL-4, IL-7 IL-9 and IL-13 (Di Santo *et al.*, 1995). This possibility seems unlikely however, as to date no functional redundancy has been demonstrated for IFN-γ.

The role of IFN-γ in resistance to R-MuLV may be clarified further by promoting expression of type 1 cytokines during infection of susceptible BALB/c mice. This may be achieved by administration of IL-12 which has been shown to promote IFN-γ expression in other models of infectious disease (Gately *et al.*, 1993; Gazzinelli *et al.*, 1994a; Oswald *et al.*, 1994a; Sieling *et al.*, 1994). IFN-α also promotes the expression of predominantly type 1 cytokines (Finkelman *et al.*, 1991) which raises the possibility that the limitation of R-MuLV infection of BALB/c mice by administration of IFN-α (Hekman and Trapman, 1985; Ruprecht *et al.*, 1990) may be due at least in part to elevated production of type 1 cytokines. In this context it is also notable that Johnson *et al.*, (1988) found that direct administration of IFN-γ to susceptible Swiss mice with established F-MuLV infection led to permanent disease regression in a significant proportion of treated mice. It may be interesting, in this light, to examine the effects of IFN-γ, IL-12 or IFN-α administration upon the course of R-MuLV infection.

Hom *et al.*, (1991) showed that both $CD4^+$ and $CD8^+$ T cells were required for optimal acquired resistance to secondary challenge with R-MuLV. In this chapter, the role of these T cell subsets in the pathogenesis of a primary R-MuLV infection was investigated. The depletion of BALB/c or C57BL/6 mice of $CD4^+$ T cells was found to partially ameliorate the splenomegaly associated with R-MuLV infection. This finding was consistent in 2 experiments and suggests that $CD4^+$ T cells may be required for optimal virus growth and disease pathology. This observation is similar to those made by Yetter *et al.*, (1988) who showed that development of disease (MAIDS) during LP-BM5 MuLV infection of C57BL/6 mice is dependent upon $CD4^+$ T cells.

The role of $CD4^+$ T cells in the pathogenesis of R-MuLV disease is unclear, however a number of possible functions can be postulated. Firstly, $CD4^+$ T cells may be a major site of early virus replication and, without these cells, the progression of disease is partially inhibited. This hypothesis seems unlikely given the lack of F-MuLV replication in spleen T cells as reported by Isaak *et al.*, (1979). This issue may be resolved by determining the level of R-MuLV infection of $CD4^+$ cells. Secondly, $CD4^+$ T cells may be required to allow the replication of R-MuLV within non-$CD4^+$ target cells. $CD4^+$ T cells influence the proliferation and state of activation of a number of cell types such as macrophages and B cells through cell surface interactions and the secretion of cytokines. $CD4^+$ T cells have also been shown to produce cytokines involved in erythropoiesis ie. IL-3 and GM-CSF (Mosmann and Coffman, 1989; Kelso *et al.*, 1991) which may facilitate the proliferation of R-MuLV infected progenitor erythroid cells. Thus, depletion of $CD4^+$ T cells may inhibit the pathogenesis of R-MuLV by preventing the activation of target cells. In this context it interesting to note that the requirement for $CD4^+$ T cells in the development of MAIDS is dependent upon their cell surface interaction with B cells which are the primary target of virus infection (Giese *et al.*, 1994; Morse *et al.*, 1995).

A third possible role for $CD4^+$ T cells in the pathogenesis of R-MuLV may be their mediation of inappropriate anti-viral immune responses at the cost of protective immune responses. Depletion of $CD4^+$ T cells has been shown to modulate the type of immune response generated towards protein antigen (Field *et al.*, 1992) or infectious agents (Sadick *et al.*, 1987; Müller *et al.*, 1993). In this context, the removal of $CD4^+$ cells from R-MuLV infected mice may ablate the generation of an ineffective immune response and allow the expression of a protective immune response. It would therefore be of interest to compare levels of antiviral antibodies and CTL in intact and $CD4^+$ T cell depleted mice. $CD4^+$ T cells also mediate inflammation in a number of model systems. It may be that depletion of these cells reduces the influx of cells into the spleen thereby reducing the mass of the spleens in R-MuLV infected BALB/c and C57BL/6 mice. In light of this possibility, it would be important to

compare the structure and cellular composition of the spleen of intact or $CD4^+$ T cell depleted mice infected with R-MuLV. Furthermore, alternative measures of virus growth such infectious virus in the spleen or in plasma as well as viral antigen in mice depleted of $CD4^+$ T cells may provide a greater insight into the role of these cells in pathogenesis of R-MuLV infection.

The depletion of $CD8^+$ T cells from C57BL/6 mice enhanced the development of splenomegaly associated with R-MuLV infection. Resistance to a wide range of viral infections has been shown to be dependent upon $CD8^+$ T cells. These include vaccinia (Ruby and Ramshaw, 1991), ectromelia (Buller *et al.*, 1987a) and HSV (Nash *et al.*, 1987) infections. Furthermore, the high incidence of recovery from F-MuLV in *Fv-2*$^{s/s}$, *H-2*^{b} mice requires the generation of cytotoxic $CD8^+$ T cells (Chesebro and Wehrly, 1978; Earl *et al.*, 1986; Chesebro, 1990; Robertson *et al.*, 1992). These observations raise the possibility that the increased splenomegaly following R-MuLV infection of C57BL/6 mice depleted of $CD8^+$ cells may be due to the loss of antiviral $CD8^+$ CTL. It would be interesting, therefore, to determine if antiviral $CD8^+$ CTL are generated in resistant C57BL/6 and susceptible BALB/c mice. Depletion of $CD8^+$ cells from BALB/c mice enhanced R-MuLV induced splenomegaly (experiment 1, Table 3.9). This observation suggests that these cells may, to some extent, limit R-MuLV disease progression in susceptible mice. However, a subsequent experiment suggested that $CD8^+$ cells may play only a limited role in the control of R-MuLV infection (experiment 2, Table 3.9). The reason for these disparate observations are unclear however flow cytometry analysis demonstrated that the efficiency of depletion was approximately equal in both experiments. Furthermore, the effects of CD8 depletion on R-MuLV induced splenomegaly in C57BL/6 mice were similar in both experiments. Clearly further experiments were required to resolve the role of $CD8^+$ cells in R-MuLV infection of BALB/c mice.

In summary, the data presented in this chapter indicate that both BALB/c and C57BL/6 mice predominantly produce type 1 cytokines following R-MuLV infection. Neutralisation of different cytokines with

monoclonal antibodies suggested that a number of these factors were not essential for resistance or susceptibility to R-MuLV. Depletion of T cell subsets suggested that $CD4^+$ T cells may be required for optimal growth of R-MuLV in C57BL/6 and BALB/c mice.

Chapter 4

The role of nitric oxide in the pathogenesis of R-MuLV infection

4.1 INTRODUCTION

The experiments described in this chapter examine the role of NO in R-MuLV infection of mice. NO has emerged as a major effector molecule in immunity to tumours (Drapier and Hibbs Jr, 1986; Stuehr and Nathan, 1989; Kwon *et al.*, 1991), bacteria (Stuehr and Marletta, 1987), parasites (Adams *et al.*, 1990; Green *et al.*, 1990; Wei *et al.*, 1995) and helminths (James and J., 1989). Recently, it was shown that NO can also act as an antiviral effector molecule. Production of NO by IFN-γ-activated macrophages was found to inhibit replication of HSV-1, ectromelia and vaccinia viruses in these cells (Croen, 1993; Karupiah *et al.*, 1993b). The effect of NO on retrovirus replication is largely unexplored, however NO was recently found to inhibit replication of F-MuLV *in vitro* and *in vivo* (Akarid *et al.*, 1995). Other reports indicate that peptides encoded by HIV induce macrophages to release NO (Mollace *et al.*, 1993; Pietraforte *et al.*, 1994) which may have protective consequences or may contribute to neuropathology associated with HIV disease (Dawson *et al.*, 1993; Hayman *et al.*, 1993).

NO has also been shown to mediate some of the pathology associated with infectious diseases. This includes the severe hypotension found in cases of septic shock and TNF-α therapy (Kilbourn *et al.*, 1990a; Petros *et al.*, 1991) and the profound immunosuppression observed during some parasitic infestations (Candolfi *et al.*, 1994; Rockett *et al.*, 1994a) and bacterial infections (Al-Ramadi *et al.*, 1992; Gregory *et al.*, 1993). In these cases, lymphocyte proliferation was found to be markedly suppressed, due, at least in part, to NO. Although the exact mechanism of NO-mediated inhibition of lymphocyte proliferation is unclear, it has been postulated that the inhibition of key enzymes and other mechanisms (see Section 1.5.5) may suppress lymphocyte proliferation (Rockett *et al.*, 1994a).

R-MuLV infection of susceptible mice is characterised by uncontrolled virus growth and erythroproliferation while in resistant mice, virus growth and proliferation of progenitor erythroid cells is limited. Given

the antiviral and antiproliferative properties of NO outlined above (see also Section 1.5.5), it is possible NO may inhibit virus replication and cellular proliferation in mice resistant to R-MuLV. In addition, NO production by infected mice may suppress potentially protective antiviral immune responses. The experiments described in this chapter were designed to address potential antiviral, antitumour and immunosuppressive properties of NO during R-MuLV infection.

4.2 MATERIALS AND METHODS

4.2.1

Mice	As described in Section 2.2.1
Virus	As described in Section 2.2.2
Virus titration	As described in Section 2.2.5
R-MuLV ELISA	As described in Section 2.2.7
Statistics	As described in Section 2.2.15

4.2.2 RT-PCR detection of iNOS mRNA

Messenger RNA was extracted from spleen cells of uninfected BALB/c or C57BL/6 mice or mice infected with R-MuLV as described in Section 3.2.8. cDNA was generated from 1 µg of total spleen cell RNA as described in Section 3.2.9. Inducible nitric oxide synthase cDNA was detected using PCR amplification with iNOS specific primers (Appendix 1; Oswald *et al.*, 1994b). The primers were designed to span at least one intron to discriminate between amplification of cDNA and any contaminating genomic DNA.

Amplification of iNOS cDNA was performed using reagents described in Section 3.2.10. After incubation of the reaction mixture for 3 min at 94°C, cDNA was amplified using the following conditions: denaturation at 95°C for 5 sec, primer annealing at 60°C for 5 sec and primer extension at 72°C for 1 min. The cDNA was amplified for between 25 and 35 cycles. Amplified cDNA products were analysed by electrophoresis through 1.5% agarose gels and visualised by ethidium bromide staining (Sambrook *et al.*, 1989).

To ensure that the iNOS RT-PCR procedure was specific, RNA was extracted from RAW 264.7 cells (American Type Culture Collection, MD, USA) which are known to transcribe iNOS mRNA following stimulation with LPS. Prior to RNA extraction, the cells were stimulated with 1 µg/ml LPS (*E. coli* 0128:B12) for 6 h to induce iNOS mRNA expression.

RNA was isolated as described in Section 3.2.8. Total RNA from stimulated RAW 264.7 cells was reverse transcribed and amplified by PCR as described above.

RAW 264.7 cells were maintained in RPMI supplemented with 10% FCS, 10 mM HEPES, 2 mM L-glutamine and antibiotics. Cells were passaged every 2 days at a 1:10 dilution.

4.2.3 Assay for Reactive Nitrogen Intermediates

Reactive nitrogen intermediates (RNI) nitrate and nitrite are stable oxidation products of NO (Marletta *et al.*, 1988). As an measure of NO production, RNI were measured using the Griess assay (Rockett *et al.*, 1994b). The level of nitrite in 30 µl of culture supernatant or mouse plasma was determined. In each test, samples were diluted in 20 µl of water and reacted with 100 µl of Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dihydrochloride [Sigma] in 1 M H_2SO_4). Protein was precipitated by the addition of 100 µl of 10% trichloroacetic acid and removed by centrifugation at 12,000g for 5 min. The absorbance of each supernatant was measured at 540 nm, with a reference at 650 nm, using a microplate reader (Molecular Devices). To assay nitrate levels, nitrate was first reduced to nitrite. This was achieved by the addition of 5 µl nitrate reductase (0.025 Units) and 15 µl reduced β-nicotinamide adenine dinucleotide phosphate (NADPH, 20 µg; Boehringer Mannheim, Mannheim, Germany) to 30 µl of sample. The reaction was incubated at room temperature for 20 min. The amount of reduced nitrate was determined as for nitrite (described above) but without dilution of samples with water. Known amounts of sodium nitrate or sodium nitrite diluted in MLCM or normal mouse plasma were used as standards for the calculation of levels of nitrite or nitrate.

4.2.4 Plasma RNI determination

Blood was obtained from mice via cardiac puncture or from tail vein bleeding. Mice bled by cardiac puncture were first anaesthetised with 500 µl of avertin (2% 2-methyl-2-butanol, 2% 2,2,2,-tribromoethanol; Fluka) given i.p. Blood from either source was collected, placed into a

microcentrifuge tube containing heparin sulphate (5 U in 10 µl per 500 µl blood) and stored on ice. Blood cells were collected by centrifugation at 12,000g for 5 min at 4°C. The plasma was removed, aliquoted and stored at -70°C until assay for RNI.

4.2.5 Spleen cell RNI production

Single cell suspensions were prepared from pooled spleens of mice as described in Section 2.2.9. NO production by spleen cells was determined following stimulation with LPS *in vitro*. Cells were cultured at $1.6x10^6$ cells/well in flat bottomed 96 well plates (Nunc) in MLCM containing 1 µg/ml LPS (*E.coli* strain 0128:B12, water phenol extracted; Sigma). After 72 h, plates were centrifuged at 200g for 5 min and supernatants were removed and aliquoted. The samples were stored at -20°C until assayed for RNI concentration as described in Section 4.2.3.

4.2.6 Production of RNI by T and B cell proliferation assays

The production of NO by spleen cells under the conditions used in proliferation assays was determined. Spleen cells were prepared as described in Section 2.2.9. $2x10^5$ spleen cells/well were cultured with immobilised anti-CD3 antibodies or LPS as described in Section 2.2.10. After 72 h, the plates were centrifuged at 200g for 2 min, supernatants were removed and stored at -20°C. RNI content was assayed as described in Section 4.2.3.

L-N-methyl arginine (L-NMA) was added to T and B cell proliferation assays in order to block the production of NO. Spleen cells were cultured at $2x10^5$ cells/well in MLCM containing various concentrations of L-NMA (prepared and generously provided Mr M. Rolph, Division of Cell Biology, JCSMR) and stimulated with immobilised anti-CD3 antibodies or 10 µg/ml LPS as described in Section 2.2.10. RNI accumulation was measured in supernatants taken after 72 h culture. Proliferation was determined by $[^3H]$-TdR incorporation as described in Section 2.2.10.

In order to inhibit the production of prostaglandins, indomethacin was added to LPS-stimulated, spleen B cell proliferation assays. Cells were

cultured using conditions described in Section 2.2.10 in the presence of various concentrations of indomethacin. Proliferation was measured by [^{3}H]-TdR incorporation as described in Section 2.2.10.

4.2.7 L-NMA therapy of R-MuLV infection

BALB/c or C57BL/6 mice were used to examine the effects of L-NMA therapy on R-MuLV infection. Mice of each strain were infected with 10^4 pfu R-MuLV i.p. in gelatin saline or were given gelatin saline only. Half of the infected and uninfected mice were injected twice daily with L-NMA (2 mg in 200 µl normal saline) i.p. and were given water containing 4 mg/ml L-NMA. The remaining mice were given water without additives and injected twice daily with normal saline. Treatment with L-NMA was started 8 h prior to infection with R-MuLV. After 14 days of therapy, plasma was taken from each mouse as described in Section 4.2.3 and stored at -70°C until assay for plasma RNI or R-MuLV. Spleens were also taken and weighed in tared sterile tubes containing 5 ml H16/5% FCS. Single cell suspensions were then prepared from each spleen and assayed for ecotropic virus expression using the XC infectious centre assay described in Section 2.2.6.

4.2.8 TNF-α therapy of R-MuLV infection

Recombinant murine TNF-α (rMu TNF-α) was generously provided by Genentech. The specific activity of rMu TNF-α was 10^7 U/mg. For injection into mice, stock rMu TNF-α was diluted in sterile PBS and administered in a volume of 200 µl i.v. Control mice were injected with PBS alone. In some experiments mice were bled 10 h later by tail vein. Plasma was isolated as described in Section 4.2.4. and subsequently assayed for RNI. The effect of TNF therapy on virus growth was measured by determining spleen weight 3 days after rMu-TNF-α injection and assaying viral antigen in plasma taken at the same time.

4.3 RESULTS

4.3.1 Comparative analysis of inducible nitric oxide synthase (iNOS) mRNA expression by spleen cells

RT-PCR amplification was used to determine the levels of mRNA encoding iNOS in the spleen after infection with R-MuLV (Figure 4.1a). Equivalent levels of iNOS mRNA expression were found in spleen cells from uninfected BALB/c and C57BL/6 mice. R-MuLV infection of BALB/c mice induced iNOS mRNA expression at 1 week p.i. which increased with time to levels approximately 10 fold above normal levels by 3 weeks p.i. Expression of iNOS mRNA by spleen cells from R-MuLV infected C57BL/6 mice was elevated at 2 and 3 weeks p.i. and reached levels approximately 10 times those of cells from uninfected mice at 3 weeks p.i. Amplification through 27 cycles of PCR yielded much less product DNA than after 30 cycles which indicates that PCR amplification of iNOS cDNA was logarithmic under the conditions used.

To demonstrate that the amplification of iNOS cDNA was proportional to the amount of input cDNA and to the number of cycles of amplification, serial 10 fold dilutions of cDNA known to contain iNOS mRNA (equivalent to between 5000 and 5 pg total RNA) were amplified through 25, 30 or 35 cycles of PCR. Amplification of relatively high levels of input iNOS cDNA (5 ng total RNA) led rapidly to non-logarithmic amplification which yields only slight increases in product DNA with increasing numbers of cycles of amplification (Figure 4.1b). Low levels of iNOS cDNA (5 pg total RNA) were readily detected after 35 cycles of amplification.

4.3.2 Plasma RNI in R-MuLV infected mice

In vivo, NO is readily oxidised to form the RNIs, nitrate and nitrite (Marletta *et al.,* 1988). The accumulation of RNI in the plasma of mice has been successfully used to measure NO production in number of model systems. The levels of RNI in the plasma of R-MuLV infected

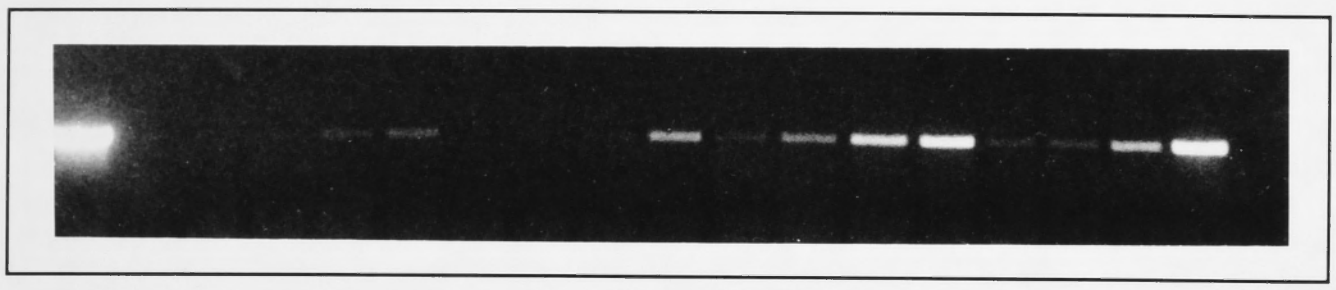
Figure 4.1 ***RT-PCR analysis of iNOS mRNA expression during R-MuLV infection***

RNA was extracted from LPS stimulated RAW 267.4 cells and spleen cells of BALB/c and C57BL/6 mice which were uninfected or infected with R-MuLV 1, 2 or 3 weeks earlier. iNOS mRNA was detected by RT-PCR amplification. PCR products were electrophoresed through 1.5% agarose and stained with ethidium bromide. Data shown is representative of 2 batches of RNA and at least 1 cDNA synthesis and PCR amplification of each batch of RNA.

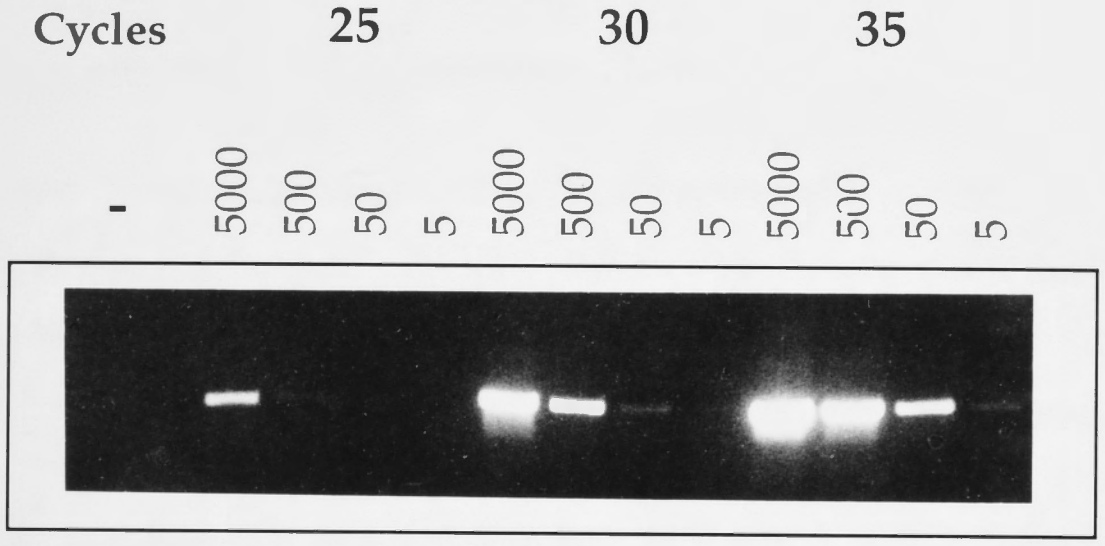
[A] RT-PCR of cDNA equivalent to 50 ng of total RNA amplified through 27 or 30 cycles of PCR.

[B] RT-PCR of LPS stimulated RAW 267.4 mRNA. cDNA equivalent to 5000, 500, 50 or 5 pg total RNA was amplified through 25, 30 or 35 cycles.

[A]


Cycles
27
30
BALB/c
C57BL/6
BALB/c
C57BL/6
+
0 1 2 3 0 1 2 3 0 1 2 3 0 1 2 3
-

[B]


Cycles
25
30
35
-
5000
500
50
5
5000
500
50
5
5000
500
50
5

mice were determined, as it was important to confirm that elevated iNOS mRNA expression resulted in NO production.

R-MuLV infection of BALB/c mice did not significantly alter plasma RNI levels during the first two weeks p.i. (Table 4.1). However, by 3 weeks p.i., plasma RNI was detected at significantly greater levels than those found in uninfected mice (Table 4.1; p=0.01). In contrast, plasma RNI levels in C57BL/6 mice did not differ significantly at each stage of infection (Table 4.1). The levels of plasma RNI found at 3 weeks p.i. were significantly greater than those found at 1 week (p=0.02) p.i., however these differences may reflect the variability of low levels of plasma RNI and are therefore unlikely to be biologically important. Plasma RNI levels in BALB/c or C57BL/6 mice were not significantly different during the first 2 weeks of infection, however, at 3 weeks p.i. plasma RNI concentrations in BALB/c mice were significantly greater than those in C57BL/6 mice (p=0.03).

4.3.3 Production of nitric oxide by splenocytes *in vitro*

It was important to demonstrate that NO could be produced by splenocytes, given the expression of iNOS mRNA by spleen cells derived from infected mice. *In vitro* NO production by LPS-stimulated spleen cells was examined using cells taken from mice at various stages of infection.

Significantly higher levels of RNI were produced by spleen cells from infected mice compared to control, uninfected animals (Table 4.2). Notably, peak production by BALB/c splenocytes occurred at 2 weeks p.i. and RNI levels were still elevated although at much lower levels at 3 weeks p.i. RNI accumulation in supernatants from C57BL/6 cells reached high levels at 2 weeks p.i., however, these levels were significantly lower compared to those found in supernatants from BALB/c cells at the same time point (p=0.04). The RNI production by C57BL/6 splenocytes was sustained to 3 weeks p.i. Spleen cells from uninfected BALB/c and C57BL/6 mice produced background levels of RNI.

Table 4.1 *Plasma RNI following R-MuLV infection*

Strain	Weeks Post Infection	Plasma RNI (μM)[a]
BALB/c	Uninfected	20.6 ± 3.7
	1	17.3 ± 4.3
	2	18.6 ± 2.7
	3	41.3 ± 5.1[bd]
C57BL/6	Uninfected	17.4 ± 3.3
	1	10.7 ± 3.2
	2	13.4 ± 4.3
	3	24.8 ± 3.9[c]

Blood was obtained from groups of 5 uninfected BALB/c or C57BL/6 mice or mice infected with 10^4 pfu R-MuLV i.p. 1, 2 or 3 weeks previously. Plasma was separated from blood cells and assayed for total RNI content. Data representative of 2 similar experiments.

a) Mean plasma reactive nitrogen intermediates of 5 mice ± SEM

b) Significant, p=0.012, when compared with plasma RNI in uninfected BALB/c mice

c) Significant, p=0.02, when compared with plasma RNI of 1 week infected C57BL/6 mice

d) Significant, p=0.03, when compared with plasma RNI of 3 week infected C57BL/6 mice

Table 4.2 ***RNI production by LPS stimulated spleen cells from R-MuLV infected mice***

Mouse Strain	Weeks Post Infection	Total RNI (μM)
BALB/c	Uninfected	3.8 ± 0.7
	1	14.6 ± 3.8[a]
	2	80.9 ± 8.8[b]
	3	25.2 ± 7.1[a]
C57BL/6	Uninfected	2.5 ± 0.5
	1	23.0 ± 6.4[a]
	2	38.6 ± 13.1
	3	48.8 ± 13.3[a]

Spleen cells were isolated from mice at various times p.i. 1.6×10^6 cells were stimulated with 1 μg/ml LPS for 72 h. Supernatants were assayed for nitrite and nitrate accumulation. Data shown represents mean total RNI in 4 wells at each time point ± SEM. Data representative of 2 similar experiments.

a) Mean total RNI significantly greater ($p<0.05$, Welch's *t* test) than mean total RNI produced by cells from uninfected mice

b) Mean total RNI significantly greater ($p<0.003$, Welch's *t* test) than mean total RNI produced by cells from uninfected mice

4.3.4 Nitric oxide is not involved in suppressed T or B cell proliferation

The expression of NO by spleen cells during R-MuLV infection raised the possibility that NO may be involved in the suppression of lymphocyte proliferation reported in Section 2.3.2. The production of NO using conditions under which lymphocyte proliferation was determined, was therefore examined. Spleen cells from uninfected or R-MuLV infected mice were stimulated with anti-CD3 or LPS as described for T and B cell proliferation assays (Section 2.2.9) and the accumulation of RNI in culture supernatants was measured. In addition, proliferation of spleen T and B cells was determined in the presence of L-NMA to inhibit NO synthesis.

R-MuLV infection had a marked effect on RNI production by spleen cells derived from both BALB/c and C57BL/6 mice following anti-CD3 stimulation of T cells (Table 4.3). At 2 weeks p.i. significant levels of RNI were found in culture supernatants while only low levels of RNI were found at 1 and 3 weeks p.i. Spleen cells from BALB/c mice produced RNI at levels similar to those of spleen cells from C57BL/6 mice at 2 weeks p.i. RNIs were not detected in supernatants from anti-CD3 stimulated spleen cells from uninfected mice.

Incorporation of L-NMA in culture media at 400 µM effectively inhibited production of NO by anti-CD3 stimulated cultures of spleen cells from infected BALB/c and C57BL/6 mice (Table 4.3). In contrast, the suppressed T cell proliferation was not ameliorated by the inclusion of L-NMA in culture media (Figure 4.2). At high concentrations, L-NMA has been shown to inhibit lymphocyte proliferation (Albina *et al.*, 1991; Schleifer and Mansfield, 1993; Gregory *et al.*, 1994), however, T cell proliferation was unaltered in the present study when L-NMA was present at concentrations which effectively inhibited RNI accumulation (400 µM, Figure 4.2; 800 µM L-NMA, data not shown). At high concentrations of L-NMA (2 mM) T cell proliferation was found to be inhibited by approximately 20 % (data not shown). These observations suggest that NO does not play a role in suppression of *in vitro* T cell proliferation.

Table 4.3 ***RNI production by anti-CD3 stimulated spleen cells from R-MuLV infected mice is inhibited by L-NMA***

Strain	Weeks Post Infection	Total RNI (μM) MLCM	Total RNI (μM) L-NMA
BALB/c	Uninfected	<2.5[a]	<2.5
	1	<2.5	<2.5
	2	16.1 ± 0.6	4.5 ± 0.1
	3	2.9 ± 0.6	<2.5
C57BL/6	Uninfected	<2.5	<2.5
	1	2.6 ± 0.4	<2.5
	2	11.8 ± 0.6	<2.5
	3	4.3 ± 0.2	<2.5

$2x10^5$ spleen cells from R-MuLV infected or uninfected BALB/c or C57BL/6 mice were stimulated with anti-CD3 antibody *in vitro*. Supernatants were taken after 72 h and assayed for RNI. Cells were cultured in MLCM or MLCM containing 400 μM L-NMA. Data points indicate mean RNI in 3 replicate wells ± SEM. Data representative of 2 similar experiments.

a) Limit of detection was 2.5 μM RNI

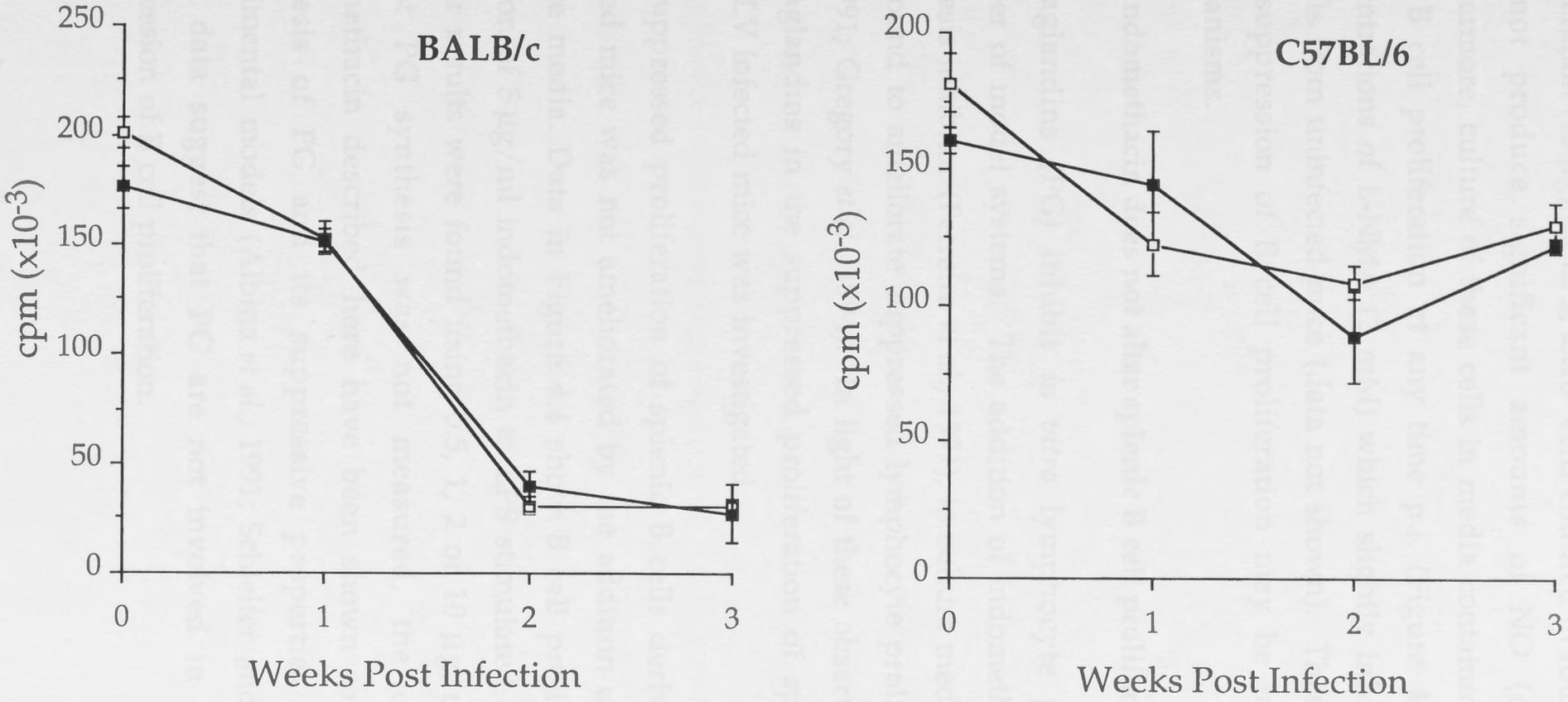


Figure 4.2 ***Effect of L-NMA on anti-CD3 stimulated spleen cell proliferation***

$2x10^5$ spleen cells from uninfected and infected BALB/c and C57BL/6 mice were stimulated with anti-CD3 antibody in MLCM (—□—) or MLCM containing 400 μM L-NMA (—■—). After 72 h, cultures were labelled with 0.5 μCi [^{3}H]-TdR for a further 16 h. Data points indicate mean proliferation of 4 replicate wells (± SEM) as measured by [^{3}H]-TdR incorporation. Data representative of 2 similar experiments.

Spleen cells cultured under the conditions used to assay B cell proliferation (10 μg/ml LPS, $2x10^5$ cells cultured in round bottomed wells) did not produce significant amounts of NO (data not shown). Furthermore, culture of these cells in media containing L-NMA did not alter B cell proliferation at any time p.i. (Figure 4.3) except at high concentrations of L-NMA (2 mM) which slightly inhibited proliferation of cells from uninfected mice (data not shown). These findings suggest that suppression of B cell proliferation may be mediated by other mechanisms.

4.3.5 Indomethacin does not alter splenic B cell proliferation

Prostaglandins (PG) inhibit *in vitro* lymphocyte proliferation in a number of model systems. The addition of indomethacin, a potent PG synthesis inhibitor (Ferreira *et al.*, 1971), to culture media in these systems was found to ameliorate suppressed lymphocyte proliferation (Albina *et al.*, 1991; Gregory *et al.*, 1994). In light of these observations, the role of prostaglandins in the suppressed proliferation of spleen B cells from R-MuLV infected mice was investigated.

The suppressed proliferation of splenic B cells derived from R-MuLV infected mice was not ameliorated by the addition of indomethacin to culture media. Data in Figure 4.4 show B cell proliferation following addition of 5 μg/ml indomethacin to LPS stimulated cultures, however, similar results were found using 0.5, 1, 2 or 10 μg/ml indomethacin. Whilst PG synthesis was not measured, the concentrations of indomethacin described here have been shown to inhibit both the synthesis of PG and its suppressive properties in a number of experimental models (Albina *et al.*, 1991; Schleifer and Mansfield, 1993). These data suggest that PG are not involved in R-MuLV induced suppression of B cell proliferation.

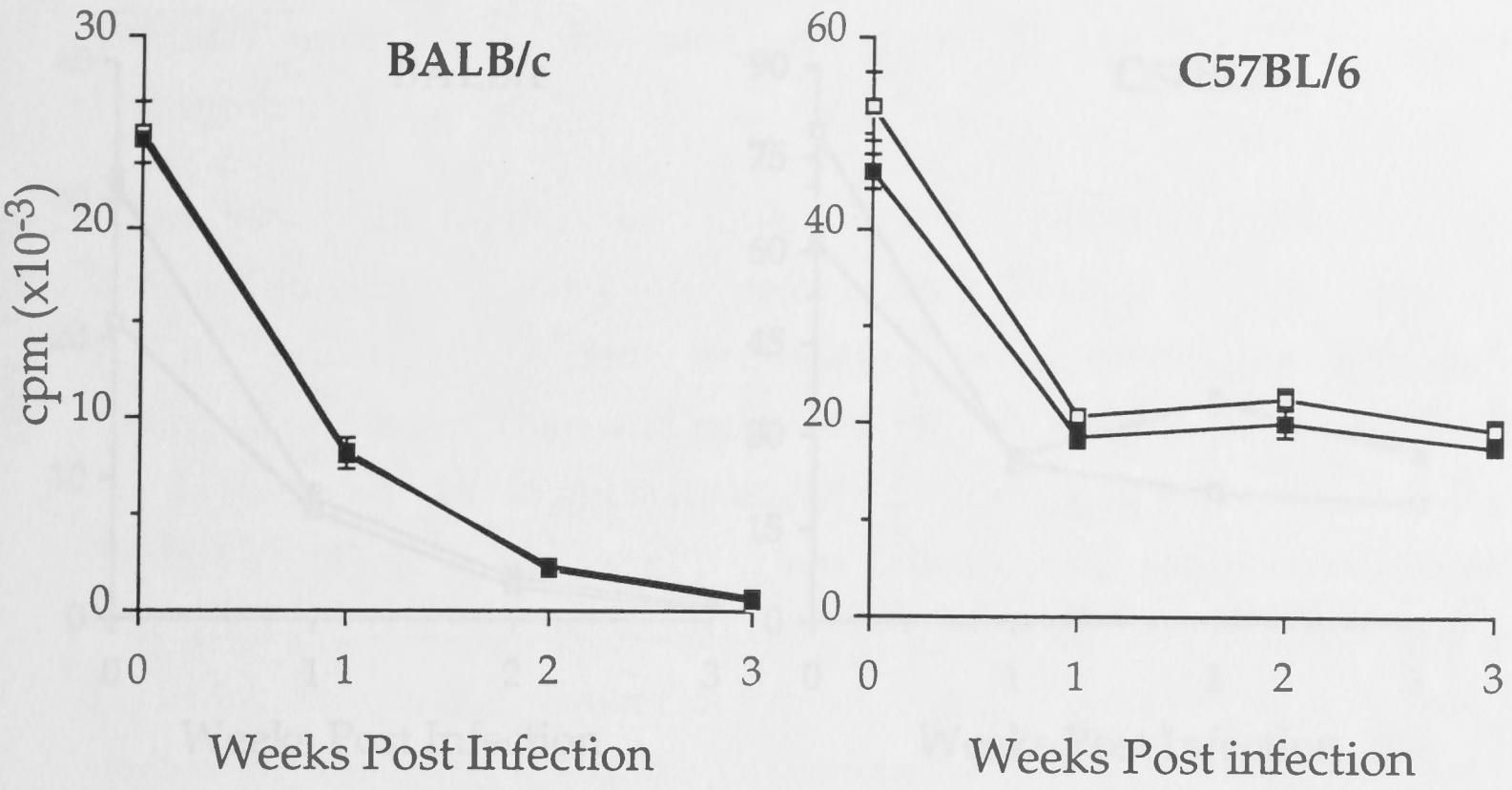


Figure 4.3 *Effect of L-NMA on LPS induced spleen cell proliferation*

$2x10^5$ spleen cells from uninfected or infected BALB/c or C57BL/6 mice were stimulated for 72 hours with 10 µg/ml LPS in the presence of 400 µMol L-NMA (—■—) or MLC M only (—□—). The cultures were labelled with 0.5 µCi $[^3H]$-TdR for a further 16 h. Data points show mean proliferation of 4 replicate cultures (± SEM) as measured by $[^3H]$-TdR incorporation. Data representative of 2 similar experiments.

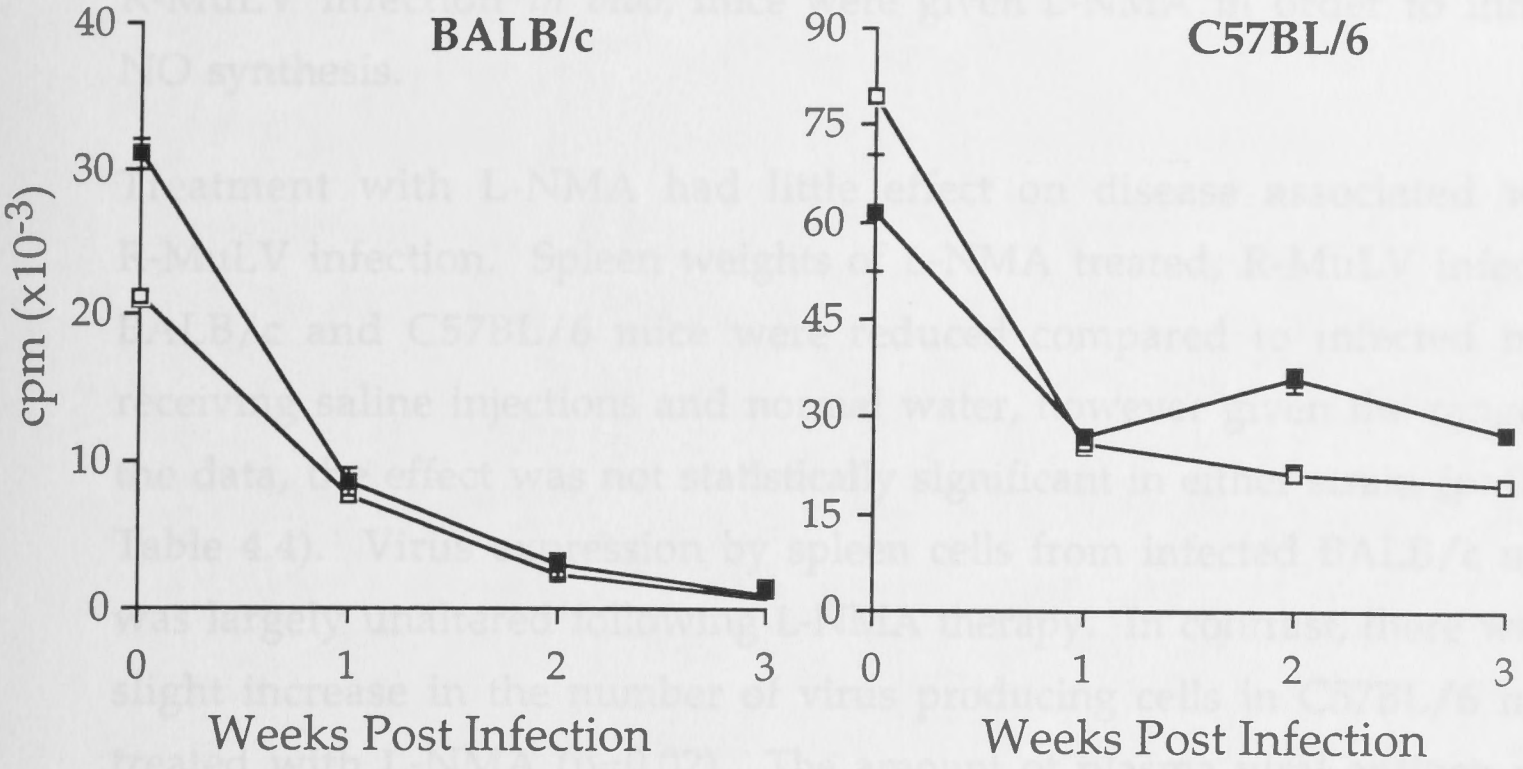


Figure 4.4 ***Effect of indomethacin on LPS induced spleen cell proliferation***

$2x10^5$ spleen cells from BALB/c or C57BL/6 mice, which were uninfected or infected with R-MuLV 1, 2 or 3 weeks earlier, were stimulated with 10 μg/ml LPS for 72 h in MLCM containing 5 μg/ml indomethacin (—■—) or MLCM alone (—□—). The cultures were labelled with 0.5 μCi $[^3H]$-TdR for a further 16 h. Data points show mean proliferation of 4 replicate cultures (± SEM) as measured by $[^3H]$-TdR incorporation. Data representative of 2 similar experiments.

4.3.6 Effect of *in vivo* L-NMA treatment on R-MuLV growth.

The data presented in Sections 4.3.2 and 4.3.3 demonstrate that NO is produced during R-MuLV infection. To explore the role of NO during R-MuLV infection *in vivo*, mice were given L-NMA in order to inhibit NO synthesis.

Treatment with L-NMA had little effect on disease associated with R-MuLV infection. Spleen weights of L-NMA treated, R-MuLV infected BALB/c and C57BL/6 mice were reduced compared to infected mice receiving saline injections and normal water, however given the range of the data, the effect was not statistically significant in either strain ($p>0.05$; Table 4.4). Virus expression by spleen cells from infected BALB/c mice was largely unaltered following L-NMA therapy. In contrast, there was a slight increase in the number of virus producing cells in C57BL/6 mice treated with L-NMA ($p=0.02$). The amount of plasma viral antigen was similar in both L-NMA treated and control mice (Table 4.4). Plasma RNI levels in mice given L-NMA therapy were significantly lower than in mice which were not treated with L-NMA (Table 4.4). This effect was independent of R-MuLV infection and was observed in mice from either strain, which suggests that L-NMA therapy inhibited NO synthesis *in vivo*. Water consumption was approximately equal in all groups of mice and was independent of L-NMA treatment or infection with R-MuLV (data not shown). Average water consumption per mouse and therefore L-NMA dose per mouse was also equivalent, however the possibility that water consumption varied between each mouse within each group cannot be excluded.

4.3.7 Effects of TNF-α on plasma RNI, splenomegaly and plasma viral antigen in R-MuLV infected mice

Johnson *et al.*, (1988) have reported that treatment with TNF-α limits F-MuLV induced disease. It was therefore of interest to determine if TNF-α also limited R-MuLV infection. As *in vivo* administration of TNF-α has been shown to induce production of NO in a number of models (Kilbourn *et al.*, 1990a; Rockett *et al.*, 1992), the observation made

Table 4.4 *Effect of L-NMA therapy on plasma RNI, development of splenomegaly and virus expression*

R-MuLV	L-NMA	Plasma RNI (μM)[a]	Spleen weight (mg)[b]	Spleen infectious centres	Plasma antigen
BALB/c					
-	-	22.1 ± 1.0	112 ± 5	-	40, 40, 40, 40
-	+	3.8 ± 0.5	146 ± 3	-	40, 40, 40, 40
+	-	18.5 ± 1.1	987 ± 170	5.65 ± 0.14	2560, 2560, 1280, 1280
+	+	5.0 ± 0.7	678 ± 36	5.46 ± 0.07	2560, 2560, 2560, 1280
C57BL/6					
-	-	7.1 ± 0.8	97 ± 11	-	40, 40, 40, 40
-	+	<2.5	72 ± 5	-	40, 40, 40, 40
+	-	12.3 ± 0.9	246 ± 37	1.07 ± 0.11	160, 80, 80, 80
+	+	<2.5	178 ± 36	1.86 ± 0.24	80, 80, 40, 40

Groups of 4 uninfected or R-MuLV infected BALB/c or C57BL/6 mice treated with either PBS or L-NMA for 2 weeks. Mice were then bled and plasma RNI and plasma viral antigen was determined. The spleen was taken from each mouse and weighed in tared, sterile tubes containing 4 ml of DMEM/5% FCS. Single cell suspensions were made from the spleen of each infected mouse and the number of infectious centres was assayed as described in the materials and methods.

a) Plasma RNI was determined 18 h after cessation of L-NMA therapy. Data represents plasma RNI of 4 mice ± SEM.

b) Mean spleen weight of 4 mice ± SEM

Table 4.4 (cont)

c) Log_{10} pfu of infectious centres producing ecotropic R-MuLV/10^7 nucleated spleen cells (mean ± SEM)

d) Endpoint of R-MuLV specific ELISA. data shown represents endpoints of individual mice

f) Similar spleen weight compared with infected mice not receiving L-NMA treatment, $p>0.15$ Welch's test

g) Similar spleen weight compared with infected mice not receiving L-NMA treatment, $p>0.15$

h) Significantly greater compared with untreated, infected mice, $p=0.02$

by Johnson *et al.*, (1988) may be due to the antiviral (Croen, 1993; Karupiah *et al.*, 1993b) or antiproliferative (Stuehr and Nathan, 1989) properties of NO. Therefore, the expression of NO following administration of TNF-α was also examined.

Recombinant murine TNF-α induced significant dose-dependent release of RNI into plasma (Table 4.5). This effect was found in both uninfected and infected BALB/c and C57BL/6 mice. Infected BALB/c mice given 500 ng of rMu TNF-α had higher plasma RNI levels than C57BL/6 mice given the same dose, the difference being significant at 7 ($p=0.015$, Welch's *t* test) and 21 days ($p=0.001$) p.i. Plasma RNI levels in C57BL/6 mice infected for 7 or 14 days were higher in mice receiving 1000 ng of rMu TNF-α compared with levels in uninfected mice ($p=0.015$, Welch's t test).

BALB/c mice infected with R-MuLV for 14 or 21 days exhibited classic symptoms of TNF toxicity, such as ruffled fur, lethargy and hypothermia of extremities, followed by death when high doses of rMu-TNF-α were given. BALB/c mice given 1000 ng or 800 ng of rMu-TNF-α at 14 days p.i. were all dead in less than 8h. In 1 of 2 experiments, 1000 ng of rMu TNF-α was also lethal for BALB/c mice at 21 days p.i. (Table 4.5, Table 4.6). In contrast, BALB/c mice which were uninfected or at 7 days p.i did not exhibit symptoms of TNF toxicity and survived following treatment with high doses of rMu TNF-α. C57BL/6 mice survived treatment with all doses of rMu TNF-α and did not exhibit symptoms associated with TNF toxicity (Table 4.5, Table 4.6). Uninfected and infected mice were both unaffected by treatment with rMu TNF-α.

Treatment of R-MuLV infected mice with rMu TNF-α had little effect on spleen weights of virus infected mice (Table 4.6). Similarly, plasma viral antigen was unaffected by rMu TNF-α therapy. Mice from both strains exhibited these phenomena. These observations suggest that treatment with rMu-TNF-α had little effect on R-MuLV replication. Furthermore, these data are consistent with NO playing a limited role in R-MuLV infection.

Table 4.5 *Plasma RNI in R-MuLV infected BALB/c and C57BL/6 mice following TNF-α therapy*

		Mean Plasma RNI (μM)	
Days Post Infection	TNF-α[a] (ng)	BALB/c	C57BL/6
Uninfected	PBS	10 ± 1	11 ± 2
	500	43 ± 14	20 ± 2[d]
	1000	62 ± 7[c]	56 ± 3[d]
7	PBS	13 ± 1	15 ± 2
	500	47 ± 7[ce]	10 ± 2
	1000	86± 17[c]	101 ± 17[cf]
14	PBS	8 ± 1	16 ± 1
	500	44 ± 7[c]	28 ± 3[d]
	800	†	ND
	1000	†	92 ± 15[cf]
21	PBS	19 ± 2	15 ± 5
	500	63 ± 6[ce]	26 ± 2
	1000	†	81 ± 40

Groups of 4 BALB/c and C57BL/6 mice which were infected with R-MuLV for 7, 14 or 21 days and then treated with a single dose of rMu TNF-α or PBS. Mice were bled 10 h later and plasma isolated. The concentration of RNI in plasma was assayed. Data shown is from a single experiment.

a) rMu TNF-α was diluted in ice cold PBS to desired concentrations and given i.v. in volumes of 200 μl.

b) Mean plasma RNI of 4 mice ± SEM

c) Significantly greater plasma RNI compared with mice given PBS ($p<0.05$ Welch's *t* test)

d) Significantly greater plasma RNI compared with mice given PBS ($p<0.02$)

Table 4.5 (cont)

e) Significantly greater plasma RNI compared with C57BL/6 mice given 500 ng rMu TNF-α, day 7 p=0.015 Welch's *t* test, day 21 (p=0,001 Student's t test)

f) Significantly greater plasma RNI compared with uninfected C57BL/6 mice given 1000 ng of rMu TNF-α (p=0.015, Welch's *t* test)

† All mice were dead in less than 10 h after injection of rMu-TNF-α

Table 4.6 *Effect of TNF-α therapy on virus growth in R-MuLV infected mice*

		BALB/c		C57BL/6	
Days Post Infection	rMu TNF-α (ng)	Spleen Weight[a]	Plasma Antigen[b]	Spleen Weight[a]	Plasma Antigen[b]
Uninfected	PBS	151 ± 5	40	104 ± 5	40
	500	175 ± 6	53 ± 13	83 ± 5	40
	800	153 ± 12	53 ± 13	85 ± 21	40
	1000	200 ± 12	53 ± 13	94 ± 4	40
10 days	PBS	277 ± 13	320	144 ± 10	40
	500	367 ± 33	480 ± 160	186 ± 5	40
	800	274 ± 21	530 ± 107	184 ± 8	40
	1000	263 ± 21	320	208 ± 10	40
17 days	PBS	1141 ± 173	1280	212 ± 53	40
	500	1032 ± 43	1707 ± 430	228 ± 25	40
	800	†	-	243 ± 40	40
	1000	†	-	291 ± 51	40
24 days	PBS	1784 ± 133	4267 ± 850	276 ± 36	40
	500	1527 ± 46	1280	265 ± 19	40
	800	1508 ± 116	5120	245 ± 24	80 ± 30
	1000	1557 ± 134	5120	305 ± 51	40

Table 4.6 (cont)

Groups of 3 BALB/c or C57BL/6 which were infected with 10^4 pfu R-MuLV for 7, 14 or 21 days were treated with various doses of rMu-TNF-α. rMu TNF-α was diluted in ice cold PBS to desired concentrations and given i.v. in volumes of 200 µl. Three days later virus growth was measured as a function of spleen weight and viral antigen in plasma. Data shown from a single experiment.

a) Mean spleen weight of 3 mice (± SEM)

b) Endpoint titre of R-MuLV ELISA of viral antigen in plasma of 3 mice (mean ± SEM)

† All mice died less than 24 h after rMu TNF-α administration

4.4 DISCUSSION

Experiments described in this chapter investigated the production of nitric oxide following R-MuLV infection of BALB/c and C57BL/6 mice. Elevated levels of mRNA encoding the NO producing enzyme, iNOS, were found in the spleens of infected BALB/c and C57BL/6 mice. Splenic iNOS mRNA expression was elevated at 2 weeks p.i. in both strains and was expressed at levels at least 8 fold greater than in uninfected mice at 3 weeks p.i. In contrast, experiments which examined the accumulation of RNI in the plasma of infected mice showed that RNI accumulation was not significantly elevated following infection of C57BL/6 mice and only significantly increased in BALB/c mice at 3 weeks p.i.

There are a number of possible explanations for the apparent contradictory results described for iNOS mRNA expression and plasma RNI accumulation. Firstly, the difference between these two results may reflect differing sensitivities of each assay system. As plasma RNI estimation measures systemic production of NO, localised production of high levels of NO, such as in individual tissues, may not induce significant increases in plasma RNI. Alternatively, excretion of RNI in urine may contribute to removal of RNI from plasma (Green *et al.*, 1981). Nevertheless, a wide range of studies have used plasma RNI levels to demonstrate NO production *in vivo*.

Secondly, iNOS expression may be controlled at a post-transcriptional level. A number of studies have demonstrated that the expression and activity of iNOS is regulated by transcriptional and post-transcriptional mechanisms (reviewed in Xie and Nathan, 1994). Cytokines are important regulators of iNOS expression and activity. Factors such as IFN-γ induce iNOS transcription (Xie *et al.*, 1992) and enhance iNOS mRNA stability (Vodovotz *et al.*, 1993). Results described in Section 3.2.2 show that IFN-γ mRNA expression by spleen cells was slightly induced during R-MuLV infection is coincident with the induction of iNOS mRNA expression in the spleen. The activity of IFN-γ in induction of iNOS expression has been shown to be cross-regulated by TGF-β which

inhibits the translation of iNOS mRNA and induces the degradation of both iNOS mRNA and protein (Vodovotz *et al.*, 1993). Although the expression of TGF-β in the spleens of R-MuLV infected mice was not investigated, it is interesting to note that infection of mice with the F-MuLV derived Fd-MIV retrovirus enhanced *in vitro* production of TGF-β (Faxvaag *et al.*, 1993). This observation raises the possibility that TGF-β expression may be elevated during murine leukemia virus infections and, as a result, limit iNOS expression and activity.

It is unclear whether the lack of increased plasma RNI is due to the inhibition of iNOS protein production or the regulation of iNOS activity. Analysis of iNOS protein expression or activity by western blot or enzymatic assay may be insufficient to resolve these questions. This is due to the regulation of iNOS activity by the availability of substrates such as L-arginine (Vodovotz *et al.*, 1994) and co-factors such as tetrahydobiopterin (Kwon *et al.*, 1989; Mellouk *et al.*, 1994) and calmodulin (Cho *et al.*, 1992). However, it remains to be shown if these potential regulatory mechanisms control NO production during R-MuLV infection.

Splenocytes from R-MuLV infected mice expressed markedly elevated levels of NO following stimulation with LPS *in vitro*. Peak *in vitro* NO production was found using spleen cells from both BALB/c and C57BL/6 mice infected 2 weeks previously. NO production by spleen cells from BALB/c mice was markedly reduced from these levels at 3 weeks p.i. while in C57BL/6 mice, levels produced at this time were similar to those found at 2 weeks p.i. These results demonstrate that during R-MuLV infection, spleen cells become activated and produce elevated levels of NO upon restimulation. This pattern of NO production by spleen cells from BALB/c mice was not in full agreement with splenic iNOS mRNA expression, as peak iNOS mRNA levels were found at 3 weeks p.i. while peak *in vitro* production occurred at 2 weeks p.i. The cause of these divergent findings is unclear, however they may reflect disparate NO inducing properties of different stimuli in each system.

The nature of the stimuli for NO production by spleen cells during R-MuLV infection is unclear. NO expression can be induced by cytokines such as IFN-γ (Ding *et al.*, 1988; Albina *et al.*, 1991) and, as discussed earlier, the levels of expression of IFN-γ mRNA by spleen cells is consistent with NO production in the spleen of infected BALB/c and C57BL/6 mice. Other cytokines, such as TNF-α, IFN-α or IFN-β, synergise with IFN-γ to augment NO production (Ding *et al.*, 1988). Expression of TNF-α mRNA was found to be elevated in the spleens of infected BALB/c mice but reduced in C57BL/6 mice (Chapter 3). These observations are consistent with a role for TNF-α in NO production in BALB/c mice and suggest that other stimuli are required for NO synthesis in C57BL/6 mice. IFN-α and IFN-β were found to elevated in the sera of C57BL/10 mice which raises the possibility that these cytokines may play a role in the induction of NO production during R-MuLV infection of the closely related C57BL/6 mice (Tóth *et al.*, 1971c). On the other hand, R-MuLV infection of BALB/c mice does not induce expression of IFN-α or IFN-β (Tóth *et al.*, 1971c).

Activated T cells have been shown to induce IFN-γ activated macrophages to produce NO via ligation of CD40 or LFA-1 molecules on the macrophage surface (Tao and Stout, 1993; Tian *et al.*, 1995). The generation of a strong anti-R-MuLV immune response in C57BL/6 mice (McCoy *et al.*, 1972) may thus induce NO production by macrophages. In contrast, BALB/c mice generate a weak anti-R-MuLV immune response (Fink and Rauscher, 1964; McCoy *et al.*, 1967; McCoy *et al.*, 1972) raising the possibility that T cell surface molecules are not major participants in NO expression by these mice.

Retrovirus encoded peptides (Hayman *et al.*, 1993; Mollace *et al.*, 1993; Pietraforte *et al.*, 1994) and tumour cells (Isobe and Nakashima, 1993; Zembala *et al.*, 1994) can induce the production of NO by macrophages in the absence of IFN-γ or LPS. As R-MuLV is expressed at high levels in the spleens of these mice (Rauscher, 1962; Boiron *et al.*, 1965 and Section 2.3.1), it is possible that the viral proteins may induce NO production by macrophages. Furthermore, R-MuLV induced tumour cells may induce

NO synthesis in BALB/c mice. As virus replication in C57BL/6 mice is somewhat limited (Rauscher, 1962; Boiron *et al.*, 1965 and Section 2.3.1) and virus induced transformation is a rare event early in infection of these mice (McCoy *et al.*, 1967), it seems likely that induction of NO production in these mice may be caused by other stimuli.

The cellular source of NO produced by spleen cells from R-MuLV infected mice is unclear. Activated macrophages have been shown to produce NO in a wide range of infectious disease models such as viral (Croen, 1993; Karupiah *et al.*, 1993b; Butz *et al.*, 1994; Rowland *et al.*, 1994) and bacterial (Al-Ramadi *et al.*, 1992; Gregory *et al.*, 1993) infections or parasitic infestations (Adams *et al.*, 1990; Liew *et al.*, 1990). Other cells such as T cells (Taylor-Robinson *et al.*, 1994) and NK cells (Cifone *et al.*, 1994) have been shown to produce NO however these examples are relatively few in number compared with reports of NO production by activated macrophages. It therefore seems likely that activated macrophages are the source of NO produced by splenocytes from R-MuLV infected mice. Future experiments which assess NO production in cultures depleted of macrophages (by adherence to plastic for example) may resolve the role of activated macrophages in NO synthesis following R-MuLV infection.

Activated macrophages have been shown to inhibit lymphocyte proliferation via the production of immunosuppressive molecules such as NO (Albina *et al.*, 1991; Al-Ramadi *et al.*, 1992). Examination of supernatants from splenic T and B cell proliferation assays showed that NO was produced by anti-CD3 but not LPS stimulated spleen cells from R-MuLV infected mice. Significant NO production by anti-CD3 stimulated cultures was found only at 2 weeks p.i. As T and B cell proliferation was inhibited at all times examined, these results suggest that NO may not directly contribute to suppressed lymphocyte proliferation in R-MuLV infected mice. This conclusion was confirmed by the failure of L-NMA to reverse the suppression of T and B cell proliferation *in vitro*. Together, these results demonstrate that NO does not play a significant role in suppressed lymphocyte proliferation during

R-MuLV infection. Similar observations have been reported in mice infected with LCMV (Butz *et al.*, 1994) or LDV (Rowland *et al.*, 1994). In these studies, spleen cells from infected mice produced high levels of NO, however NO was found to play only a minor role in the immunosuppression characteristic of LCMV or LDV infection.

Activated macrophages may also inhibit lymphocyte proliferation by the production of PG. Albina *et al.*, (1991) demonstrated that addition of thioglycolate elicited peritoneal macrophages to spleen cell cultures inhibited LPS stimulated B cell proliferation. This effect was reversible in part by the addition of the PG synthesis inhibitor, indomethacin. In the present study, addition of indomethacin to LPS stimulated spleen cell cultures failed to ameliorate suppressed B cell proliferation exhibited by spleen cells from R-MuLV mice. The expression of PG was not determined here, so it remains possible that PG production was not completely inhibited by indomethacin at the concentrations used. This seems unlikely, however, as a number of reports have shown that PG synthesis or the immunosuppressive effects of PG are reduced by indomethacin used at the range of concentrations employed here, ie. 0.5 to 10 μg/ml (Albina *et al.*, 1991; Schleifer and Mansfield, 1993). Since at these concentrations PG production should be efficiently inhibited, the suppression of B cell proliferation may be mediated by other mechanisms. Future experiments may examine the production of other inhibitory molecules such as hydrogen peroxide and TGF-β (Albina *et al.*, 1991) in suppressed lymphocyte proliferation during R-MuLV infection.

Inhibition of NO production *in vivo* using the specific inhibitor of NOS, L-NMA, had little effect on levels of viral antigen in plasma or on R-MuLV induced splenomegaly at 2 weeks p.i. Virus expression by spleen cells was unaltered by L-NMA therapy of BALB/c mice but was moderately increased in C57BL/6 mice. These observations indicate that NO does not contribute to susceptibility of BALB/c mice to R-MuLV infection or play a major role in antiviral immunity in C57BL/6 mice. These results are in contrast with observations of Akarid *et al.*, (1995) who showed that administration of L-NMA to F-MuLV-susceptible DBA/2

exacerbated F-MuLV infection. The reason for this difference is unclear, however it may reflect the much lower dose of F-MuLV given or different pathogenesis of F-MuLV in DBA/2 mice compared with R-MuLV infection of BALB/c or C57BL/6 mice. A possible explanation of the lack of effect of L-NMA upon R-MuLV infection *in vivo* may be that NO may not impair R-MuLV replication. This finding would be in marked contrast to other reports that NO limits virus replication and more recently limits F-MuLV replication (Croen, 1993; Karupiah *et al.*, 1993b; Akarid *et al.*, 1995). It will therefore, be interesting to determine if NO limits replication of R-MuLV *in vitro*.

The observation that plasma RNI was significantly suppressed in mice receiving L-NMA indicated that the protocol of L-NMA administration inhibited NO synthesis. Furthermore, Rockett *et al.*, (1992) used an identical protocol to successfully inhibit the ability of high doses of recombinant human TNF-α to induce RNI accumulation in plasma of *Plasmodium vinckei* infected mice. However, it remains possible, albeit unlikely, that the regime of L-NMA administration used here did not achieve high enough concentrations of L-NMA to inhibit NO production at various sites of virus replication. Mice with the iNOS gene disrupted have recently become available (Wei *et al.*, 1995) and may be useful in further examining the role of NO in R-MuLV infection.

Treatment of R-MuLV infected and uninfected mice with rMu TNF-α induced a rapid dose-dependent release of RNI into the plasma. This phenomenon was observed in both BALB/c and C57BL/6 mice. The ability of rMu TNF-α to induce NO synthesis *in vivo* has been demonstrated in normal mice (Kilbourn *et al.*, 1990a; Kilbourn *et al.*, 1990b) and is augmented by infection with organisms such as *P. vinckei* (Rockett *et al.*, 1992; Rockett *et al.*, 1994b) or *L. major* (Liew *et al.*, 1990). Interestingly, infection with R-MuLV also increased the release of plasma RNI following administration of rMu TNF-α compared with levels produced by uninfected mice. These observations suggest that R-MuLV infection primes or activates cells capable of producing NO in both strains of mice. This effect appears to be greater in BALB/c mice, as low doses of

rMu TNF-α (500 ng) induced higher plasma RNI release compared with similarly treated C57BL/6 mice, however this difference was only statistically significant at 1 and 3 weeks p.i. BALB/c mice which were infected for 2 or 3 weeks exhibited a marked sensitivity to the toxic effects of rMu TNF-α, with doses of 1 μg proving lethal. Uninfected BALB/c mice or those infected for 7 days survived treatment with 1 μg rMu TNF-α. C57BL/6 mice survived a dose of 1 μg rMu TNF-α at any stage of infection. It is unclear whether the toxic effects of rMu TNF-α in infected BALB/c mice are mediated by NO. It is notable that mice infected with *P. vinckei* and treated with TNF-α, LT or IL-1 develop very high levels of NO which were 5-10 fold greater than those reported in Table 4.5 (500-1000 μM plasma RNI) without significant mortality (Rockett *et al.*, 1992). Mortality associated with the administration of TNF-α to rats has been shown to be due to profound hypoglycaemia and is preventable by infusion with glucose (Chajek-Shaul *et al.*, 1990). Furthermore, *P. vinckei* infection of mice markedly enhances hypoglycaemia induced by TNF-α and IL-1 administration (Rockett *et al.*, 1994b). In light of these observations, it is likely that the lethal effects of rMu TNF-α administration to R-MuLV infected mice may not be due to NO synthesis but occurred rather as a result of impaired glucose homeostasis. The role of NO in toxicity associated with rMu TNF-α may be resolved by blocking NO synthesis with L-NMA following administration of TNF-α as described by (Rockett *et al.*, 1992).

The finding that treatment of R-MuLV infected mice with rMu TNF-α had little effect on virus growth suggests that R-MuLV may be not susceptible to the antiviral effects of TNF-α. In addition, these experiments provide further evidence that NO does not exert anti-R-MuLV effects. However, it may be that R-MuLV infection is only slightly affected by a single dose of rMu TNF-α and that multiple doses may be required for TNF-α to exert antiviral or antiproliferative effects. This contention is supported by the study of Johnson *et al.*, (1988) which showed that single high dose treatment of F-MuLV infected inbred Swiss mice reduced virus induced splenomegaly in the short term, while multiple doses over 12 days of TNF-α alone, or in combination with

IFN-γ, could induce significant long term regression in mice with established disease. In addition, the experiments reported in this chapter describe treatment of established disease with rMu TNF-α. Several studies have shown that treatment of mice with TNF-α prior to infection with HSV-1 lead to markedly prolonged survival of mice given a lethal dose of virus (Rossol-Voth *et al.*, 1991). However, pretreatment of mice with TNF-α does not protect against all viruses, as the growth of LCMV (Klavinskis *et al.*, 1989) or vaccinia virus (Lidbury *et al.*, 1993) was not altered by prophylactic treatment with this factor. Clearly, further experiments are required before a definitive statement concerning the effects of rMu TNF-α on R-MuLV infection can be made. These may include examination of the ability of TNF-α to inhibit R-MuLV replication *in vitro* or by altering the regime of TNF-α administration *in vivo* as described above.

In summary, the data presented in this chapter indicate that NO is expressed by BALB/c and C57BL/6 mice following R-MuLV infection however a role for NO in the pathogenesis of R-MuLV infection was not demonstrated.

Chapter 5

General Discussion and Concluding Remarks

5.1 GENERAL DISCUSSION

Rauscher MuLV infection of BALB/c and C57BL/6 mice produces two different disease phenotypes. BALB/c mice are highly susceptible to disease and fail to control the virus while C57BL/6 mice are resistant to disease and rapidly limit virus growth upon infection (Rauscher, 1962; Pluznik and Sachs, 1964; Boiron *et al.*, 1965 and Chapter 2). The growth of R-MuLV and the closely related F-MuLV is influenced by a number of heterologous genes (reviewed in Chesebro, 1990; Tsichilis and Lazo, 1991). Several of these genes confer resistance independently of antiviral immune responses (Mitchell and Risser, 1992). For example, *Fv-4* regulates virus entry into cells (Suzuki, 1975) while *Fv-1* restricts the integration of the viral genome into host chromosomal DNA (Gautsch *et al.*, 1978; DesGroseillers and Jolicoeur, 1983). In mice permissive to virus growth at these alleles (such as the C57BL/6 and BALB/c mice used in this study), functional T cell-dependent immune responses are required to mediate resistance to both R-MuLV (Ruprecht *et al.*, 1990; Hom *et al.*, 1991) and F-MuLV (Kitagawa *et al.*, 1986; van der Gaag and Axelrad, 1990; Robertson *et al.*, 1992) infection.

The immune response to R-MuLV exhibited by C57BL/6 and BALB/c mice has been partially characterised. Several studies have indicated that BALB/c mice exhibit a poor anti-R-MuLV immune responses following R-MuLV infection. BALB/c mice challenged with R-MuLV generated a weak CMI response to R-MuLV (Mortensen *et al.*, 1973; Peters *et al.*, 1975) and only low levels of anti-R-MuLV antibody (Tóth *et al.*, 1971a; Tóth *et al.*, 1971c). In contrast, C57BL/6 mice produce strong CMI responses as measured by the macrophage migration inhibition test (Mortensen *et al.*, 1973) and high levels of anti-R-MuLV antibody (McCoy *et al.*, 1972; Ishimoto and Ito, 1973). The aim of this thesis was to investigate the role of cytokines and $CD4^+$ and $CD8^+$ T cells in resistance or susceptibility to R-MuLV and examine the role of the recently described antiviral molecule NO in the pathogenesis of R-MuLV infection. In addition, the mechanism(s) by which R-MuLV suppresses host immune responses was investigated.

The type of immune response generated toward an infectious agent often correlates with resistance or susceptibility. The inability to control a pathogen often results from the generation of an inappropriate immune response rather the failure to generate an immune response (Bretscher *et al.*, 1992). Cytokines are integral to the generation and effector activities associated with an immune response. Moreover, the generation of certain immune responses has been associated with the production of certain patterns of cytokines (O'Garra and Murphy, 1994). Thus the production of certain patterns of cytokines may influence resistance or susceptibility to an infectious agent (Heinzel *et al.*, 1989; Heinzel *et al.*, 1991; Powrie and Coffman, 1993a). Examination of cytokine expression by spleen and lymph node cells demonstrated that predominantly type 1 cytokines were expressed following R-MuLV infection of both susceptible BALB/c and resistant C57BL/6 mice. This observation suggested that susceptibility of BALB/c mice to R-MuLV may not be due to production of an inappropriate pattern of cytokines and an ineffective immune response. Interestingly, *in vitro* restimulated spleen cells from R-MuLV infected C57BL/6 mice produced much higher levels of type 1 cytokines compared with spleen cells from infected BALB/c mice. These data raised the possibility that there may be quantitative differences in the antiviral immune response generated by these strains of mice and that insufficient IFN-γ production may lead to an immune response that cannot clear R-MuLV. Furthermore, this observation was consistent with the relatively strong anti-R-MuLV immune response generated by C57BL/6 mice compared with BALB/c mice.

However, treatment of C57BL/6 or BALB/c mice with neutralising antibodies to a number of cytokines did not affect the disease associated with R-MuLV infection in these mice. This finding suggests that these factors were not required for resistance or susceptibility to R-MuLV infection. In particular, neutralisation of IFN-γ had little effect upon R-MuLV induced splenomegaly which suggests that resistance to R-MuLV exhibited by C57BL/6 mice was not dependent upon expression of high levels of IFN-γ. In contrast, 129/Sv mice lacking functional IFN-γ receptors appeared to be more resistant to R-MuLV infection than wild type 129/Sv

mice. This finding suggested that IFN-γ activity was an important factor in susceptibility to R-MuLV exhibited by 129/Sv mice. It is unclear why IFN-γ activity appears to affect R-MuLV induced disease in 129/Sv and not in C57BL/6 or BALB/c mice. As discussed in Chapter 3, further studies are needed in IFN-γ $R^{o/o}$ mice to clarify the role of IFN-γ and other cytokines in the pathogenesis of R-MuLV infection.

The role of cytokines in the pathogenesis of another MuLV, LP-BM5, has been extensively investigated. Disease progression during LP-BM5 MuLV infection is associated with an increase in expression of type 2 cytokines and reduction in production of type 1 factors (Gazzinelli *et al.*, 1992). It was therefore postulated that type 2 cytokines were involved in progression of disease. However, further studies using anti-cytokine neutralising mAb or mice lacking functional cytokines showed that the type 2 cytokines IL-4 and IL-10 were not required for disease (Morawetz *et al.*, 1994; Uehara *et al.*, 1994; Morse *et al.*, 1995) but that IFN-γ was integral to disease progression (Gazzinelli *et al.*, 1994a; Uehara *et al.*, 1994). These studies clearly demonstrate the complexity of cytokine interaction and the necessity for experiments that can clearly define the function of individual cytokines.

A number of studies have also suggested that cytokine expression during HIV infection switches from predominantly type 1 to type 2 factors as asymptomatic infected individuals progress to AIDS (Clerici and Shearer, 1993; Clerici and Shearer, 1994). However, this is not a universal finding (Emile *et al.*, 1994; Maggi *et al.*, 1994; Romagnani *et al.*, 1994; Romagnani and Maggi, 1994). This issue may be clarified by treatment of infected individuals with recombinant cytokines and/or antibodies to cytokines. Indeed, recent clinical trials have shown that treatment of HIV infected individuals with IL-2 (Kovacs *et al.*, 1995) or antibodies to IL-6 (Emile *et al.*, 1994) partially ameliorate disease. These reports also raise the possibility that HIV infection may be successfully managed with therapy that modulates cytokine activity.

$CD4^+$ T cells were found to be required for the full development of splenomegaly associated with R-MuLV infection of BALB/c and C57BL/6 mice. The exact role of $CD4^+$ T cells in the pathogenesis of R-MuLV

infection is unclear. A number of possible functions for CD4+ T cells in R-MuLV infection may be proposed. For example, R-MuLV may replicate within CD4+ T cells and consequently, depletion of CD4+ T cells may impair virus growth. The level of infection of CD4+ T cells was not assessed, however the finding that replication of F-MuLV in T cells is limited (Isaak *et al.*, 1979) suggests that this possibility may be unlikely.

An alternative explanation may be that the depletion of CD4+ T cells may prevent the generation of an ineffective immune response. This hypothesis remains to be investigated, however it seems unlikely as depletion of CD4+ T cells limited R-MuLV induced splenomegaly in both resistant and susceptible mice. Furthermore, CD4+ T cells affect the generation and mediation of immune responses via the production of cytokines (Cher and Mosmann, 1987; Hodgkin *et al.*, 1990; Powrie and Coffman, 1993a; O'Garra and Murphy, 1994) and neutralisation of a number of cytokines known to be produced by CD4+ T cells had little effect upon the development of disease associated with R-MuLV infection. Experiments which determine the effect of depletion of CD4+ T cells upon the generation of antiviral immune responses such as antiviral CTL and antibodies may resolve the role of CD4+ T cell-dependent immune responses in resistance or susceptibility to R-MuLV.

CD4+ T cells are also required for the development of MAIDS associated with LP-BM5 infection of C57BL/6 mice (Mosier, 1984; Yetter *et al.*, 1988). Chronic activation of CD4+ T cells and their interaction with B cells, and to a lesser extent macrophages, is thought to be integral in the development of MAIDS (Giese *et al.*, 1994; Morse *et al.*, 1995). It is not precisely known how CD4+ T cells facilitate the development of MAIDS, however Giese *et al.*, (1994) recently reported that the presentation of antigen to CD4+ T cells by MHC II+ cells was required. These observations raise the possibility that CD4+ T cells may contribute to the pathogenesis of R-MuLV infection via activation of other cells. It may be speculated that CD4+ T cells may activate other cells via cytokines. Data presented in Chapter 3 did not support a role for a number of cytokines in the pathogenesis of R-MuLV. It remains possible however, that cytokines involved in haematopoiesis such as IL-3, GM-CSF and erythropoietin may promote the proliferation of

R-MuLV infected progenitor erythroid cells in the spleen of R-MuLV infected mice. It is therefore notable that activated $CD4^+$ T cells have been shown to produce IL-3, GM-CSF (Mosmann *et al.*, 1986b; Mosmann and Coffman, 1989) while activated macrophages may produce erythropoietin (Nathan, 1987; Vogt *et al.*, 1989) .

The observation that $CD4^+$ T cells were involved in splenomegaly associated with R-MuLV infection of resistant C57BL/6 mice is in contrast with the findings of Hom *et al.*, (1991) who showed that $CD4^+$ T cells from R-MuLV immune mice partially conferred resistance to naive BALB/c mice. The reason for these conflicting findings are unclear. It may be that the mechanism by which resistance is mediated in C57BL/6 mice may be different to that found in R-MuLV-immune BALB/c mice. Experiments which compare the anti-R-MuLV immune responses generated in C57BL/6 and R-MuLV immune BALB/c mice may resolve this issue.

$CD8^+$ T cells have been shown to be involved in the control of a range of virus infections such as vaccinia virus (Ruby and Ramshaw, 1991) and HSV (Nash *et al.*, 1987). C57BL/6 mice depleted of $CD8^+$ T cells were found to exhibit greater pathology during R-MuLV infection. This observation is consistent with the findings of Robertson *et al.*, (1992) who showed that recovery from F-MuLV was dependent upon $CD8^+$ T cells. In addition, the resistance to MAIDS exhibited by LP-BM5 MuLV infected A/J mice is dependent upon $CD8^+$ T cells (Makino *et al.*, 1992). From the data presented in this thesis it is unclear how $CD8^+$ T cells limit R-MuLV infection in C57BL/6 mice. $CD8^+$ T cells limit replication of other viruses by the production of antiviral cytokines such as IFN-γ (Ruby and Ramshaw, 1991) or antiviral chemokines (Cocchi et al., 1995) and/or via lysis of virus infected cells (Zinkernagel and Althage, 1977). These reports raise the possibility that these mechanisms may be involved in the control of R-MuLV infection exhibited by C57BL/6 mice. However, given that *in vivo* treatment with neutralising antibodies to antiviral factors such as IFN-γ did not alter R-MuLV infection, it seems unlikely that $CD8^+$ T cells limit virus infection by producing these cytokines. It will be therefore of interest to examine the production of chemokines and the generation of anti-R-MuLV $CD8^+$ CTL in C57BL/6 mice.

Experiments in Chapter 4 have examined the role of NO in R-MuLV infection of BALB/c and C57BL/6 mice. iNOS mRNA was found to be expressed in the spleen of R-MuLV infected BALB/c and C57BL/6 mice. In addition, spleen cells from R-MuLV infected mice also exhibited a marked ability to synthesise NO. Given that NO has been shown to limit the replication of a number of viruses (Croen, 1993; Karupiah *et al.*, 1993b; Harris *et al.*, 1995; Karupiah and Harris, 1995) and impair proliferation of tumour cells (Drapier and Hibbs Jr, 1986; Kwon *et al.*, 1991; Lepoivre *et al.*, 1991) and bone marrow cells (Punjabi *et al.*, 1992), it seemed likely that NO would be involved in the control of R-MuLV. Furthermore, it was recently reported that NO produced by activated macrophages could impair F-MuLV infection *in vitro* and NO may limit F-MuLV replication *in vivo* (Akarid *et al.*, 1995). However, similar levels of expression iNOS mRNA and NO by spleen cells from both susceptible and resistant mice following R-MuLV infection. Furthermore, inhibition of NO synthesis *in vivo* by treatment of infected BALB/c or C57BL/6 mice with the iNOS inhibitor L-NMA did not affect virus growth. Together, these observations suggest that NO may not play a role in resistance or susceptibility to R-MuLV. It remains possible that the use of competitive inhibitors of NOS did not completely prevent the production of significant levels of NO. Mice with the iNOS gene disrupted have recently become available (Wei *et al.*, 1995) and may help to clarify further the role of NO in R-MuLV infection.

The role of NO in other retroviral infections such as HIV is unclear. *In vitro* studies show that HIV proteins stimulate human macrophages to produce NO (Mollace *et al.*, 1993; Pietraforte *et al.*, 1994). Cultures of human astrocytes or glial cells have been shown to produce NO following exposure to HIV peptides. These findings raise the possibility that NO may play a role in the neuropathology associated with HIV infection (Dawson *et al.*, 1993; Mollace *et al.*, 1993; Koka *et al.*, 1995). However, examination of RNI levels in cerebrospinal fluid from people at various stages of HIV infection found no evidence for increased NO production by neural tissue (Milstein *et al.*, 1994). The expression of NO in HIV infection is unclear as contradictory studies show that serum RNI levels are unchanged (Evans *et*

al., 1994) or elevated (Zangerle *et al.*, 1995) in HIV infected people. It will be interesting to determine if HIV replication *in vitro* and in HIV infected individuals is inhibited by NO.

A feature of R-MuLV infection of susceptible mice with R-MuLV is the marked suppression of both antibody production (Siegel and Morton, 1966; Ceglowski and Friedman, 1968; Millian and Schaeffer, 1968; Seidel and Lauenstein, 1969) and CMI responses (Gabrilovich *et al.*, 1994). Resistant strains of mice such as C57BL/6 mice also exhibit suppressed antibody production following R-MuLV infection, however this effect is slight and transient (Seidel and Lauenstein, 1969) while the development of CMI is unaltered (Mortensen *et al.*, 1973). The role of immunosuppression in the pathogenesis of viral infections is intriguing. While many retroviruses suppress immune responses toward retroviral and heterologous antigens (Bendinelli *et al.*, 1985; Chesebro, 1990) and inhibit resistance to secondary infections (Buller *et al.*, 1987b; Moors *et al.*, 1990), the importance of immunosuppression to retrovirus persistence remains unclear. The correlation of the degree of R-MuLV-induced immunosuppression with the level of virus growth in C57BL/6 or BALB/c mice suggests that the suppression of potentially protective immune responses may facilitate viral persistence. Furthermore, Moloney MuLV infection is exacerbated in BALB/c mice co-infected with R-MuLV (Chirigos *et al.*, 1968). However, (Morrison *et al.*, 1986) found that F-MuLV infected A.BY mice ($H\text{-}2^{b/b}$, $Rfv\text{-}3^{s/s}$) had high titres of circulating virus without exhibiting the severe immunosuppression found in congenic A/WySn mice ($H\text{-}2^{a/a}$, $Rfv\text{-}3^{s/s}$). Further studies indicate that F-MuLV induced immunosuppression is regulated by loci within the *H-2D* subregion while resistance to infection was associated with genes within the *H-2K* or *I-A* subregions (Morrison *et al.*, 1987; Chesebro, 1990). These findings show that immunosuppression is not essential in the pathogenesis of F-MuLV and, by analogy, raises the possibility that the resistance or susceptibility to R-MuLV may be independent of R-MuLV-associated immunosuppression.

Nevertheless, immunosuppression associated with retroviral infection is of increasing interest in light of the HIV pandemic. Experiments in this thesis were designed to explore the immunosuppression associated with

R-MuLV infection. A number of potential mechanisms by which R-MuLV induces immunosuppression may be proposed. R-MuLV infection of susceptible BALB/c mice leads to a marked accumulation of progenitor erythroid cells in the spleen. This observation raised the possibility that suppressed lymphocyte proliferation described in Chapter 2 may be due to decreased proportions of lymphocytes in the spleen of infected mice. Examination of the proportion of T and B cells in the spleen of R-MuLV infected mice suggested that a reduction in the proportion of immunoreactive cells could not completely account for the immunosuppression associated with R-MuLV infection.

Infection of immune cells such as T cells, B cells and macrophages is another possible cause of immunosuppression associated with retroviral infections (Bendinelli *et al.*, 1985). The role of virus infection of immune cells in R-MuLV-associated immunosuppression was not investigated in this thesis. F-MuLV replicates in B cells and to a lesser extent in T cells of infected mice (Isaak *et al.*, 1979). This observation raises the possibility that R-MuLV induced immunosuppression may be due at least in part to infection of lymphocytes.

Further experiments described in this thesis also showed that spleen cells from R-MuLV infected BALB/c or C57BL/6 mice suppressed the proliferation of splenic T cells from uninfected mice. These data suggest that spleen cells from infected mice produce an immunosuppressive factor. The identity of this suppressive factor was not characterised, however similar co-culture experiments using spleen cells from F-MuLV indicate that F-MuLV is capable of suppressing immune cell function *in vitro*. This finding raises the possibility that R-MuLV itself is the suppressive factor described above. It would be interesting to determine if infection with R-MuLV is required for this effect. Alternatively, retroviral proteins may be responsible for the suppression described above as there are numerous reports that retrovirus encoded peptides can suppress immune responses (Fowler *et al.*, 1977; Snyderman and Ciancolo, 1984; Orosz *et al.*, 1985; Ruegg *et al.*, 1989a).

Since IL-2 acts as an autocrine and paracrine T cell growth factor (Fraser *et al.*, 1993; Seder *et al.*, 1994), deficient IL-2 production is a potential mechanism of suppressed T cell proliferation. Indeed, suppressed IL-2 production is associated with the impaired T cell proliferation which is a feature of infection with viruses such as LCMV (Saron *et al.*, 1990) and MCMV (Blackett and Mims, 1988) and retroviruses including LP-BM5 MuLV (Gazzinelli *et al.*, 1992), F-MuLV (Lopez-Cepero *et al.*, 1988) and HIV (Murray *et al.*, 1984). However, in the examples cited above, T cell proliferation was not restored by the addition of exogenous IL-2 suggesting that the immunosuppression was not at the level of IL-2 production. In Chapter 3, *in vitro* IL-2 production by spleen cells from R-MuLV infected mice was unaltered except late in infection while proliferation splenic T cells from both BALB/c and C57BL/6 mice was impaired at all times p.i. examined. These observations are consistent with the hypothesis that suppressed T cell proliferation found in R-MuLV infected mice was independent of IL-2 production. The possibility that defective expression of IL-2 receptor or impaired signalling following IL-2 receptor ligation may account for suppressed T cell proliferation is not excluded by this data. These avenues of investigation may clarify the role of IL-2 in suppressed T cell proliferation associated with R-MuLV infection.

Immunosuppression associated with infectious disease may also be due to suppressive effects of host derived molecules. Activated macrophages have been shown to produce immunosuppressive molecules such as NO and PG (Nathan, 1987; Albina *et al.*, 1991). The immunosuppression induced by NO is due to a vigorous immune response involving the production of high levels of IFN-γ (Al-Ramadi *et al.*, 1992; Schleifer and Mansfield, 1993). Although IFN-γ and NO were produced by spleen cells from R-MuLV infected mice, NO was not involved in suppressed T or B cell proliferation. These findings are similar to infection with LCMV (Butz *et al.*, 1994) or LDV (Rowland *et al.*, 1994), where NO is produced by activated macrophages but does not cause the immunosuppression associated with these infections. Prostaglandin production by macrophages has also been shown to impair B cell proliferation (Albina *et al.*, 1991), PG were found to play little role in the suppressed B cell

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APPENDIX 1

Primer sequences used for PCR amplification of cDNA

Cytokine	Primer	Primer Sequence	Bases Spanned[a]	Reference
IFN-γ	IFN γ 5'	5'-TGA ACG CTA CAC ACT GCA TCT TGG-3'	71-94	Clonetech
	IFN γ 3'	5'-CGA CTC CTT TTC CGC TTC CTG AG-3'	508-530	"
TNF-α	TNF S	5'-GGC AGG TCT ACT TTG GAG TCA TTG C-3'	820-843	Murray *et al.*, (1990)
	TNF AS	5'-ACA TTC GAG GCT CCA GTG AAT TCG G-3'	1102-1127	"
IL-2	IL-2 S	5'-TTG ATG GAC CTA CAG GAG CTC CTG AGC-3'	201-227	Morris *et al.*, (1995)
	IL-2 AS	5'-AGA GAG CCT TAT GTG TTG TAA GCA GGA GG-3'	565-592	"
IL-4	IL-4 S	5'-GAA TGT ACC AGG AGC CAT ATC-3'	100-121	Svetic *et al.*, (1991)
	IL-4 AS	5'-CTC AGT ACT ACG AGT AAT CCA-3'	463-485	"
HPRT	HPRT S	5'-GTT GGA TAC AGG CCA GAC TTT GTT G-3'	514-538	"
	HPRT AS	5'-GAT TCA ACT TGC GCT CAT CTT AGG C-3'	652-678	"
iNOS	iNOS S	5'-CCC TTC CGA AGT TTC TGG CAG CAG-3'	3199-3223	Oswald *et al.*, (1994b)
	iNOS AS	5'-GGC TGT CAG AGC CTC GTG GCT TTG G-3'	3671-3695	"

Primer sequences were obtained from previously published or commercial sources. All primers were synthesised by the using standard phosphoramidite chemistry (Applied Biosystems, NJ, USA) at the Biomolecular Resources Facility of the JCSMR. Interferon-γ primers were originally purchased from Clonetech Laboratories (Palo Alto, CA, USA) and subsequently synthesised by the JCSMR BRF.

a) Numbering of sequences based on the following reports: IFN-γ (Gray and Goeddel, 1983); TNF-α (Fransen *et al.*, 1985); IL-2 (Kashima *et al.*, 1985); IL-4 (Lee *et al.*, 1987); HPRT (Konecki *et al.*, 1982), iNOS (Xie *et al.*, 1992).